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MICROBORE COLUMNS PACKED WITH GRAPHITIZED CARBON BLACK FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The preparation of short microbore columns (0.5 mm I.D.) packed with commercially available carbon black is described. The chromatographic and packing performances of these columns were evaluated with different eluites and eluents. A comparison with larger diameter columns packed with the same material showed that microbore columns yield a higher efficiency (reduced plate height between 2.5 and 3.5) and consume less solvent. Some practical applications of pharmaceutical interest indicate that the use of microbore columns allows the rapid and selective elution of relatively high-molecular-weight compounds. The possibility of using pure, volatile solvents as eluents makes these columns particularly suitable for combined liquid chromatography–mass spectrometry applications.

INTRODUCTION

In recent years, increasing interest has been devoted to the development and use of microcolumns in high-performance liquid chromatography (HPLC)[1–5]. These columns, which have been classified[6] as open-tubular, packed microcapillaries and microbore columns, depending on the preparation procedure and the inner diameter, offer some real advantages with respect to the conventional HPLC columns commonly used in routine separations[2,3,6]. Low solvent consumption, small amounts of packing material, high theoretical efficiency and the possibility of direct connection to a mass spectrometer[7] make microcolumns promising and attractive for the separation and identification of the components present in complex organic mixtures. Although open-tubular columns[8,9] and, to some extent, packed microcapillaries[10] have been shown to yield high efficiency coupled with good permeability, micropacked and packed capillaries seem to offer, at present, some attractive features because their higher sample capacity[2] allows the use of the miniaturized devices available today. Their lower efficiency, on the other hand, can be partly or completely counterbalanced by the use of more selective stationary phases as packing materials. An example is the use of carbon-based materials, which behave as natural reversed phases[11–13]. Among these materials has been examined graphitized carbon black (GCB), already extensively used in gas chromatography (GC) for its outstanding

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properties such as homogeneity, chemical structure and lack of pores. Owing to its inertness, it can also be used with a wide variety of solvents, this aspect being critical when microcolumns are connected to a mass spectrometer. As highly volatile eluents (such as pentane, dichloromethane and acetonitrile) can be used in combination with GCB columns, rapid and efficient volatilization of the sample can be achieved in the ion source of the mass spectrometer. It is worth remembering that, by choosing a suitable solvent, it would be possible to use the eluent itself as an ionizing medium to obtain mass spectra by chemical ionization.

This paper describes the preparation of short (20–40 cm) microbore columns (0.5 mm I.D.) packed with Carbopack B particles having a mean diameter ranging between 25 and 33 μm. The efficiency of these columns has been evaluated in terms of the eluotropic strength of the eluent, the capacity ratio of the solute (k') and the amount of substance injected. A comparison between microbore and larger diameter columns (1.6 mm I.D.) indicates that the former are more efficient, require smaller flow-rates and give shorter analysis times than the latter. Preliminary applications carried out with pure eluents suitable for combined HPLC–mass spectrometry (MS) indicate that these microbore columns are suitable for the rapid elution of organic mixtures containing relatively high-molecular-weight components.

EXPERIMENTAL

Column preparation

Carbopack B (80–100 mesh) supplied by Supelco (Bellefonte, PA, U.S.A.) was used for preparing the microbore columns. The original material, which has a surface area of *ca.* 80 m^2/g, was first ground on a mechanical sieving machine by using rubber balls. The fraction between 100 and 5 μm was further screened on metal screens of suitable size by washing the particles for 30 min with 1 l of acetone. The operation was repeated three or four times until the particles formed by simple agglomeration of microparticulate matter were disrupted and the fine dust was removed. After the removal of the solvent, the resulting material was dried and screened until the particles present in the various fractions appeared well defined and with an almost spherical shape under a microscope. Particles with diameters between 25 and 33 μm were used for packing glass columns 20–40 cm in length and with inner diameters ranging between 0.4 and 0.5 mm. As the outer diameter of the glass tubes was 6 mm, the connection of the columns with both the injector and the detector was made by using commercially available fittings.

The columns were filled with carbon particles by using the dry packing technique commonly used in GC. Small portions of packing were slowly inserted into the inlet of the column, which was constantly vibrated. During the packing, the column outlet was closed with a 5 μm metal screen and connected to an aspirating water pump. Further arrangement of the packing material was obtained by using pentane as liquid eluent and slightly increasing the inlet pressure of the column until a flow-rate of 0.2 ml/min was achieved. After 1 h the column was ready for use.

Apparatus and materials

To obtain a low dead volume at the column inlet, a home-made injector similar to that previously described[12] was employed. This device permits the observation of

the exact point where the sample is injected (usually 0.5 mm above the carbon particles). This technique, equivalent to the on-column injection adopted for capillary columns, prevents excessive band spreading of the sample if small volumes are injected with sufficient velocity. The columns were set into a Varian Model 4100 liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with a syringe solvent delivery system. In some instances (low flow-rates), a home-made apparatus consisting of a metal reservoir in which the eluent was pressurized by a gas tank was used in place of the Varian liquid chromatograph. A Variscan variable-wavelength UV detector equipped with an 8-μl detector cell was connected to the column outlet by using a 2 cm long metal tube of I.D. 0.1 mm. When a make-up fluid was used to reduce the band spreading within the detector, a small-volume microvalve (Precision Sampling, Baton Rouge, LA, U.S.A.) was inserted between the column outlet and the UV detector via a low-dead-volume three-way connector.

All solvents and chemicals were obtained from Carlo Erba (Milan, Italy). The eluents were of HPLC grade, whereas benzene, 1,2,3-trimethylbenzene, 1,2,4,5-tetramethylbenzene, acenaphthene, hexamethylbenzene and hexachlorobenzene were of GC grade, with less than 1% of total impurities.

Testosterone, epitestosterone and Δ^4-androstene-3,17-dione were supplied by Supelchem (Milan, Italy). The other compounds were obtained from various sources.

Standard solutions containing 20 μg/μl of each component were used for measuring the column efficiency. The volume injected ranged between 0.5 and 0.1 μl.

RESULTS AND DISCUSSION

Fig. 1 shows the height equivalent to a theoretical plate (HETP) values measured on microbore carbon columns when different test compounds and eluents were used. The test mixtures were selected in order to cover the widest range of capacity ratio whereas the eluents were chosen on the basis of their eluotropic strength. According to the calculations based on the solvophobic theory[14] and the dispersive solubility parameters[15], the four solvents selected cover the range of eluotropic strength where most of the practical applications are likely to be carried out. The common trend observed with the various eluents indicates that the column efficiency is a function of the relative retention. This suggests that band spreading may take place in either the injection or detection system. Experiments carried out by adding a make-up liquid at the detector inlet showed that, by reducing the residence time in the detector by a factor ten, a substantial improvement in the column performances (between 30 and 20%) can be obtained for compounds showing relatively low retentions (between $k' = 0$ and 5). However, above this value of the capacity ratio the HETP values measured without make-up fluid were, within experimental error, equal to those reported in Fig. 1. As the efficiency measured by reducing the residence time in the detector was still dependent on the retention, we believe that additional diffusion was also occurring in our injection system (low injection speed) or in the connecting lines.

Whatever is the reason for this dependence, it is reasonable to assume that the lowest value of HETP (H_{min}) measured in the range of k' between *ca.* 4 and 15 and under the experimental conditions used for generating the curves in Fig. 1 is very close to the maximum efficiency that can be obtained with these columns.

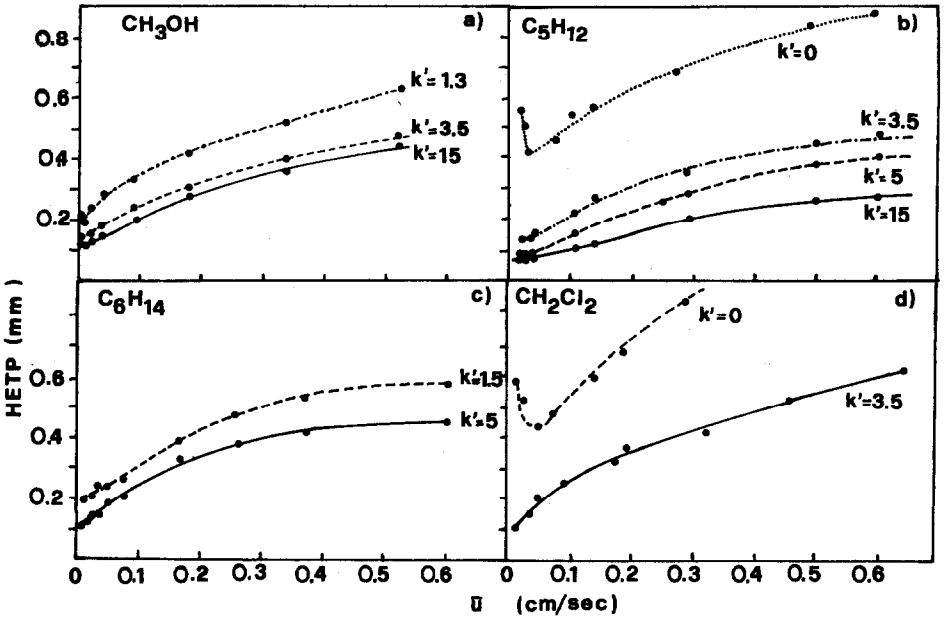

Fig. 1. Plots of HETP *vs.* linear velocity for microbore columns (25 cm × 0.5 mm I.D.) packed with Carbopack B (25–33 μm). (a) Eluent: methanol. Eluites: -·-·-, 1,2,3-trimethylbenzene; - - -, 1,2,4,5-tetramethylbenzene; ——, acenaphthene. (b) Eluent: *n*-pentane. Eluites: ..., benzene; -·-·-, acenaphthene; - - -, hexamethylbenzene; ——, hexachlorobenzene. (c)Eluent: *n*-hexane. Eluites: ——, hexachlorobenzene; - - -, hexamethylbenzene. (d) Eluent: dichloromethane. Eluites: - - -, benzene; ——, hexachlorobenzene.

As the values of H_{min} range between 2.5 (pentane, $k' = 15$) and 3.5 (methanol, $k' = 15$) times the average particle diameter (which is, in our case, *ca.* 30 μm) and the theoretical value expected on the basis of kinetic considerations[16] is twice the particle diameter ($H_{min} = 2d_p$), we can conclude that short microbore carbon packed columns are sufficiently efficient to be used for practical separations.

This conclusion is also supported by the results shown in Fig. 2a, where the efficiency of microbore columns is compared with that measured on conventional columns (1.6–2 mm) packed with carbon particles having the same mean diameter. For the sake of clarity, the results obtained for a solute having $k' = 5$ are reported together with the pressure drop and flow-rate measured at a value of the linear velocity (0.5 m/sec) where both columns exhibit a readily measurable impedance. The data shown in Fig. 2a indicate that the efficiency of columns having larger diameters is smaller than that measured on microbore columns, whereas the flow-rate and pressure drop are higher. Whereas the difference in flow-rate is substantial and leads to a 10-fold decrease in solvent consumption, the increase in permeability is not as good as that observed with packed microcapillary columns (0.1 mm I.D.)[10]. Measurements of the column resistance factor φ' (defined as $\varphi' = \Delta P d_p^2 / Lu\eta$ where ΔP is the pressure drop in atm × 10^6, L is the column length in cm, u is the linear velocity in cm/sec, η the eluent viscosity in poise and d_p the particle diameter in cm) carried out on six different columns indicate that microbore columns packed with carbon exhibit a permeability slightly higher than packed columns ($\varphi'_{micro} \approx 700$, $\varphi'_{packed} = 850$). Based on these values, the minimum value of the Knox separation

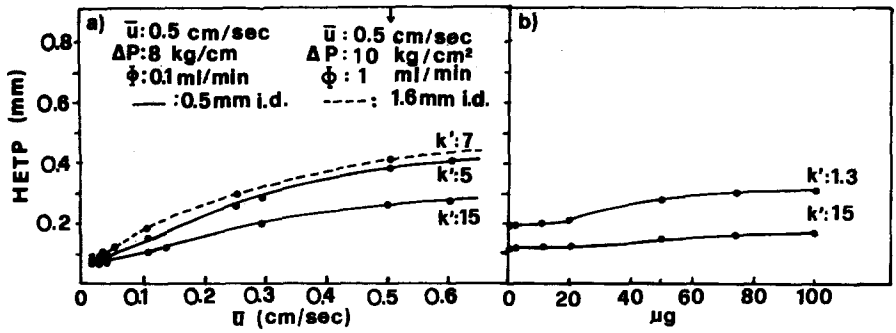

Fig. 2. (a) HETP *vs.* linear velocity plots obtained with columns having different inner diameters. – – –, 40 cm × 1.6 mm I.D. column packed with Carbopack B (25–33 μm). Eluent: *n*-pentane. Eluite: 3,6-dimethylnaphthalene. ——, 40 cm × 0.5 mm microbore column packed with Carbopack B (25–33 μm). Eluent: *n*-pentane. Eluites: hexamethylbenzene (k' = 5), acenaphthene (k' = 15). (b) Change in the minimum HETP *vs.* amount injected into a microbore column (25 cm × 0.5 mm I.D.) packed with Carbopack B (25–33 μm). Eluent: methanol; linear velocity, 0.05 cm/sec. Eluite: 1,2,4,5-tetramethylbenzene (k' = 1.3), hexamethylbenzene (k' = 15).

impedance E ($E = h_{min}^2\varphi'$ where $h_{min} = H_{min}/d_p$), which evaluates the column performance in terms of plate number, analysis time and pressure drop required for a given separation[16], can be estimated to be of the order of 4000–6000. As this value is similar to that measured on conventional columns but two or three times greater than that found with packed microcapillary columns[10], we can conclude that there is no great advantage in making microbore columns packed with carbon longer than 1 m when the inner diameter is 0.5 mm.

Fig. 2b shows the change in H_{min} as a function of increasing amounts of substance injected into the column. As can be seen, for compounds having a linear adsorption isotherm, the capacity of microbore columns packed with GCB is fairly good and allows the separation of the components present in a mixture at submilligram levels without severe losses in efficiency.

The results shown are not sufficient, however, to decide whether microcolumns packed with GCB are capable of satisfying some basic requirements which makes their practical use advantageous. First, it is important to know if such columns exhibit enough resolving power for separating those compounds which cannot be determined (the sample undergoes thermal decomposition) or are difficult to separate by gas chromatography (the analysis time is long and the resolution poor). The second requirement is that such a resolving power should be obtained in a short time, with a single-solvent eluent and at relatively low flow-rates. Compliance with these last three conditions is very important in routine work as a large number of analyses can be performed with low solvent consumption and simple and inexpensive apparatus can be used.

In order to answer the first question, it is useful to evaluate the extent to which the selectivity, the capacity ratio of the solutes and the number of theoretical plates affect the chromatographic resolution of a carbon packed column. As the smallest carbon particle size that can be obtained with sufficient reproducibility is of the order of 20 μm, the selectivity of the carbon surface must be at least twice that observed on chemically bonded phases (CBP), where particle sizes of 5 μm are commonly used.

Only in this case will a comparable chromatographic resolution be obtained for solutes exhibiting capacity ratios larger than *ca.* 6. Indeed, recent studies carried out on different carbonaceous packings[11–14,17,18] clearly show that the interaction between a solute molecule and the flat and rigid carbon surface is very strong and higher than that occurring with the mobile hydrocarbonaceous ligands bonded to the silica surface. As a result, columns packed with GCB are much more selective than those packed with CBP but a higher retention of the solute is observed. Consequently, in order to achieve rapid elution of those relatively high-molecular-weight compounds which need to be separated by HPLC, it is often necessary to use strong, non-polar eluents (such as those reported in Fig. 1) in combination with high linear flow-rates. However, the use of high flow-rates becomes really advantageous only if the decrease in the number of theoretical plates does not significantly affect the chromatographic resolution. By looking at the slopes of the curves in Fig. 1 we can say that, for the type of microcolumns investigated here, the use of relatively high flow-rates allows the best compromise between resolution and analysis time to be made. For this reason, it can be estimated that, for many practical purposes, the optimal flow-rates through the column are those ranging between 20 and 100 μl/min when the capacity ratio of the solute is between 6 and 10.

The chromatograms in Fig. 3a and b, obtained by eluting the same test mixture at two different linear flow-rates, illustrate this point well. While the time required for the elution of acenaphthene and hexamethylbenzene increases by a factor 20 when the linear flow-rate is decreased from 1 to 0.04 cm/sec, the resolution measured at the lower linear flow-rate is only twice the resolution measured at the highest flow-rate that can be passed through the column.

Figs. 4 and 5 show some examples of the separations that can be obtained when microbore columns packed with GCB are used under the experimental conditions discussed above.

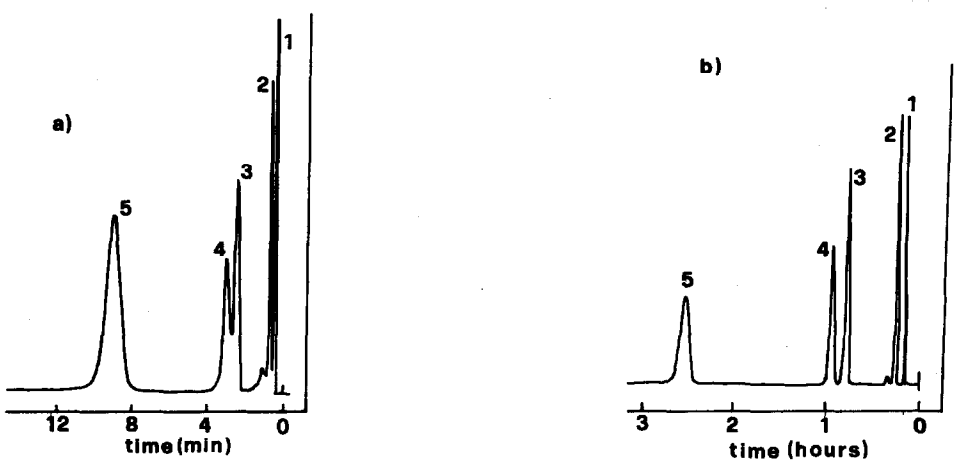

Fig. 3. Chromatograms of a test mixture containing (1) benzene, (2) 1,2,4,5-tetramethylbenzene, (3) acenaphthene, (4) hexamethylbenzene, (5) hexachlorobenzene, eluted on a 25 cm × 0.5 mm I.D. microbore column packed with Carbopack B working at two different linear flow-rates: (a) u = 1 cm/sec; (b) u = 0.04 cm/sec. Eluent: *n*-pentane.

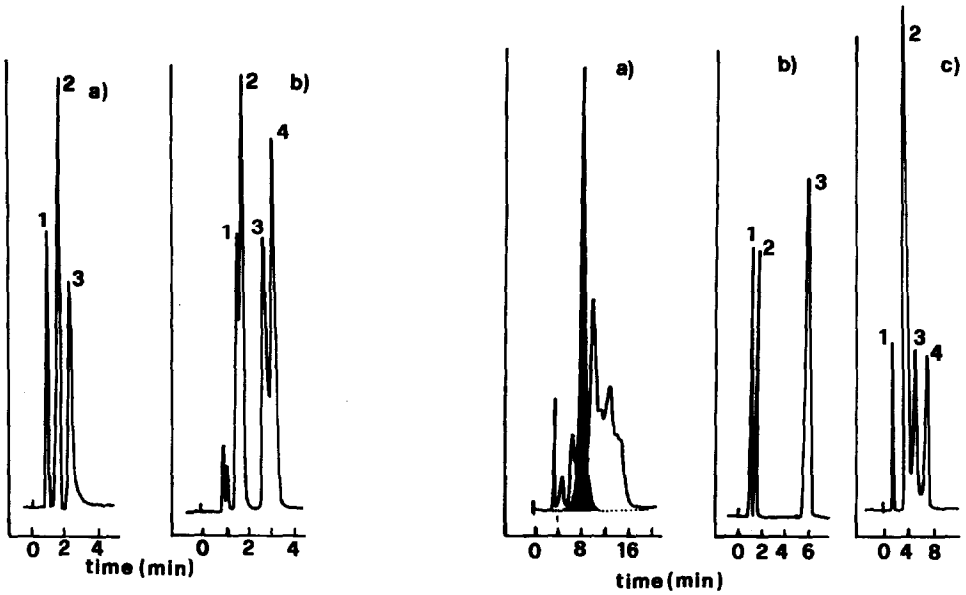

Fig. 4. (a) Separation of an artificial mixture containing (1) epitestosterone, (2) testosterone and (3) Δ^4-androstene-3,17-dione, obtained on a 25 cm × 0.5 mm I.D. microbore column packed with Carbopack B (25–33 μm). Eluent: *n*-hexane; flow-rate, 0.1 ml/min. (b) analysis of a pharmaceutical preparation containing (1) testosterone propionate, (2) testosterone isocaproate, (3) testosterone phenylpropionate and (4) testosterone decanoate, eluted on a 40 cm × 0.5 mm I.D. microbore column packed with Carbopack B (25–33 μm). Eluent: dichloromethane; flow-rate, 0.1 ml/min.

Fig. 5. (a) Analysis of a reaction mixture prepared for the synthesis of 4-cholesten-3-one (the corresponding peak is indicated by the shadowed area). Microbore column (25 cm × 0.5 mm I.D.) packed with Carbopack B (25–33 μm). Eluent: dichloromethane; flow-rate, 50 μl/min. (b) Separation of the components present in an analgesic preparation: (1) phenylacetamide; (2) phenacetin; (3) caffeine. Microbore column (40 cm × 0.5 mm I.D.) packed with Carbopack B (25–33 μm). Eluent: methanol; flow-rate, 0.1 ml/min. (c) Separation of an artificial mixture of anti-inflammatory steroids commonly used in pharmaceutical preparations: (1) fluocinolone acetonide; (2) dexamethasone; (3) cortisol; (4) cortisone. Conditions and column as in (b).

Fig. 4a shows the complete separation of an artificial mixture containing epitestosterone (Δ^4-androsten-17-α-ol-3-one), testosterone (Δ^4-androsten-17β-ol-3-one) and Δ^4-androstene-3,17-dione and Fig. 4b shows the separation of a pharmaceutical preparation containing testosterone propionate, isocaproate, decanoate and phenylpropionate.

Fig. 5a shows the separation of a reaction mixture containing the various isomeric species formed during the synthesis of 4-cholesten-3-one. In this instance the column was also used for the enrichment and purification of the sample to be identified by MS. Fig. 5b shows the separation of the components present in an analgesic preparation containing phenylacetamide, phenacetin and caffeine. Fig. 5c shows the chromatogram of an artificial mixture containing some steroids commonly used in anti-inflammatory pharmaceutical preparations. The compounds eluted were fluocinolone acetonide, dexamethasone, cortisol and cortisone. Triamcinolone, which is not shown, is also eluted by the column (the retention time is about 10 min) but gives a large tailed peak which is not useful for quantitative determination.

It is important to note that the separations shown in Figs. 4 and 5 were all performed with single-solvent, slightly polar eluents characterized by relatively high volatility. The use of aqueous solutions, buffers and binary-solvent eluents containing liquid modifiers[19] was intentionally avoided in view of the possible combination of these columns with a mass spectrometer.

CONCLUSIONS

Commercially available GCB can be used successfully for the preparation of microbore columns in HPLC. These columns are more efficient than the conventional ones, require less solvent and make possible the rapid elution of relatively high-molecular-weight compounds from carbon-based materials. The possibility of using a wide variety of pure and volatile solvents can be particularly advantageous in view of the direct combination of these columns with a mass spectrometer. Further investigations are needed in order to increase the permeability of microbore columns by decreasing their inner diameter to 0.1 mm (packed microcapillaries). This would allow shorter analysis times coupled with the possibility of making longer and more efficient columns.

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