PROTECTION, EFFICIENCY, AND SOME PRACTICAL ASPECTS OF USE OF CGC COLUMNS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Some practical adaptations of compact glass cartridge (CGC) columns and design of related components of the high-performance liquid chromatographic system are suggested; included are the design of a Teflon guard column and its filling procedure, an intra-column sample injector for on-column sample delivery or for injection into the precolumn, replacement of a contaminated packing or replenishment of packing at the top of the CGC column, a procedure for shortening the column to a desired length, and the possibility of thermostatting the column by means of a water jacket. These adaptations make it possible to increase the lifetime of the CGC column as much as five times without loss of efficiency, and to use partly damaged or imperfectly packed columns.

High performance liquid chromatography (HPLC) is today the most progressive separation method enjoying widespread use[1]. Considerable attention is being paid to the problem of the optimum design of the chromatographic column which is the heart of the chromatographic system[2]. The requirements placed on an HPLC system comprise not only a rapid and efficient separation but also easy handling, replacement, and protection of the column as well as its long lifetime and availability at a reasonable price[3]. These requirements are met by the so-called compact glass cartridge (CGC) columns, produced, among other manufacturers, also by Laboratorní Přístroje works in Prague. The assets of the design of CGC columns and their performance have been discussed recently[3,4], little attention, however, has been paid to the lifetime aspect of CGC column handling.

In the present paper, some adaptations suitable for the long-term use of CGC columns are described, *viz.*, the design of a guard column and the way of its filling, an intra-column sample injector, partial replacement of contaminated packing or replenishment of packing at the column top, a procedure for shortening packed CGC columns to any desired length, and CGC column thermostatting.

EXPERIMENTAL

Apparatus. Used were a DuPont 848 liquid chromatograph (DuPont Instruments, U.S.A.), a Rheodyne 7120 injector with a 50 μl loop, a 254 nm fixed wavelength UV detector (DuPont,

U.S.A.), a Pye Unicam 4020 variable-wavelength UV detector (Pye Unicam, U.K.), a BAS LC 4A electrochemical detector (Bioanalytical Systems, U.S.A.) equipped with a glassy carbon electrode (0·72 V *vs* Ag/AgCl electrode), and an OH 814/1 recorder (Radelkis, Budapest). A 50 × 4·6 mm presaturation column[5] packed with Silasorb silica gel 30 μm particle size (Lachema, Brno) was inserted between the pump and injector. The CGC columns were used as received, *i.e.* 150 × × 3·2 mm dimensions packed with Separon SIX C18 (Laboratorní přístroje, Prague), or shortened and adapted as given below.

Chemicals. The eluting solutions were prepared from chemicals of reagent grade purity (Fluka, Buchs, and Lachema, Brno); acetonitrile (Koch-Light, U.K.) was used without purification; water was redistilled from a $KMnO_4$ solution. The eluents were degassed in vacuum and filtered through a 0·22 μm membrane filter (Millipore, U.S.A.).

Guard column design and filling. Fig. 1 shows the guard column directly linked to the CGC column, fastened with a nut functioning as the intra-column sample injector. To the metal jacket **3** of the guard column are attached sealing nuts **2** and **5**; nut **5** connects the metal jacket **3** of the precolumn to the jacket **7** of the column. In jacket **3** is freely inserted the body of the guard column, 2–3 cm long, consisting of a steel jacket **12** with a pressed-in Teflon tube **13**, 3 mm i.d.; on its bottom is a steel or Nylon screen **14** with a metal ring **15** and a Teflon washer **16**. The guard column is linked to the CGC column *via* a union **6**. In place of nut **1**, a commercial nut with fastening of the inlet capillary can be used for sealing the system. The outer jacket of the CGC column is fitted with nipples **8** for attachment to a thermostat. The guard column was filled by the modified procedure[6]: the dry packing (d_p = 10 μm) is poured into the column by means of a microfunnel, and a drop of methanol is added and allowed to soak in. The packing is rammed with a steel piston whose diameter matches exactly the column; the piston is slowly rotated while removing it from the column and next batch of the dry packing is added. The whole procedure is carried out 2–4 times and the packing is accomplished within 15–20 min. The precolumn is usable for 30–50 analyses of samples from biological material.

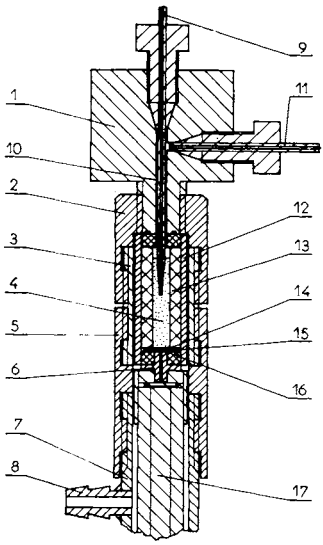

FIG. 1

Design of the guard column and intra-column injector directly linked to the CGC column. **1** inlet nut of the intra-column injector, **2** sealing nut, **3** jacket of the guard column with a thread, **4** precolumn packing, **5** sealing nut, **6** union, **7** jacket of the CGC column, **8** nipple for attachment of a water thermostat, **9** capillary for sample feeding from the loop injector, **10** axial drilling, **11** capillary for mobile phase feeding, **12** steel jacket of the precolumn, **13** Teflon tubing, **14** steel screen, **15** metal ring, **16** Teflon washer, **17** CGC column

Design of the intra-column sample injector. The injector (Fig. 1) consists of an inlet nut **1** with axial drilling **10** in which a capillary **9** for sample delivery from the injection device is fastened. The outer diameter of the capillary is smaller than the axial drilling diameter, and the capillary is so long that it penetrates 5—8 mm into the packing **4**. The axial drilling also contains the orifice of capillary **11** through which the pure eluting solution is fed onto the top of the packing. The ratio of the flow rates in the two capillaries is controlled by means of two independent pumps or one pump fitted with a splitter.

Adaptation of the inlet of used CGC *columns and shortening of packed columns.* After removing the Teflon washer and steel screen 1·5—2 cm of the contaminated packing was discarded. The screen was cut to match the column diameter and inserted into the tube. A new machined Teflon ring with a hole matching the inner column diameter was pressed into the metal ring. The empty space was packed with the reversed phase (d_p = 10 μm) by applying the same procedure as used for the filling of the guard column.

The CGC column to be shortened was first washed with redistilled water and then shortened by conventional cutting (making a groove by means of a diamond or a file) as with any other glass tube. With this procedure, the wetted packing tears exactly in the place where the glass is cut and the entire chromatographic column remains undisturbed. The cut end of the tube was ground with a fine carborundum grinder also on the sides, and the metal ring was sealed to it with epoxy resin. After the seal set, the contaminated packing layer (maximum 1 mm) was replaced with a fresh one, the steel screen was placed on it and the Teflon ring was pressed in. The outer aluminium or brass jacket was so adapted that it was 1 cm shorter than the shortened column; the other components were used without modifications.

RESULTS AND DISCUSSION

Fig. 2 shows a typical dependence of the mobile phase flow rate on time for a new but improperly packed CGC column. The flow rate at a constant pressure of 30 MPa decreased in 25 h from the initial 1·154 ml min^{-1} to 0·74 ml min^{-1}, *i.e.*, by 36%; The volume of eluent that passed through the column within that time was 1 400 ml.

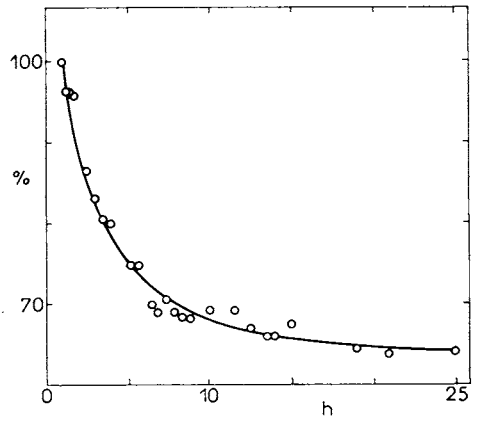

FIG. 2

Time dependence of the mobile phase flow rate for a new but poorly packed CGC column. Packing: Separon SIX C18, d_p = 5 μm, mobile phase: methanol–water 1 : 3; ΔP = 30 MPa

no sample was injected and a virtually pulse-free mode was attained with the high-pressure pump used (Haskel, model 28030). Still, the packing column was 5–7 mm reduced in height. Clearly, with such a dead volume the sample separation is deteriorated and the column usually has to be replaced. We adapted the top part of the column as described in Experimental and used it for the analysis of a mixture of nucleosides and bases. The example shown in Fig. 3 gives evidence that the CGC column with the internal guard precolumn can be used for the separation of multicomponent mixtures without considerable loss of efficiency. According to the manufacturer's certificate, a new column at $k' = 3{\cdot}31$ has an efficiency of 7 560 theoretical plates, whereas the value for the adapted column at $k' = 3{\cdot}26$ (adenosine) measured was 6 500 theoretical plates, *i.e.* 14% less. This value includes also the contributions from the less effective sample delivery through the loop injector and from the use of the more complicated retention mechanism. In the literature[7] a 10–20% decrease in efficiency is reported for columns packed with spherical silica gel after 20–30 analyses, which agrees well with our measurements.

With a well packed CGC column, however, it is not convenient or necessary to intervene into the chromatographic bed in this manner; the much more efficient approach consists in the use of an external guard column. This arrangement is particularly suitable for analyses of samples from biological material, with a risk of a rapid degradation of the analytical column, or for the preconcentration of highly dilute samples. Fig. 4 shows chromatograms from the separation of monoamines and their metabolites on a CGC column to which a 2 cm guard column was interfaced. Fig. 4*a* corresponds to a case where the guarding effect of the precolumn was disturbed, whereupon peak height lowering, formation of bumps and double peaks, tailing, and extended retention times resulted. Fig. 4*b* pertains to a newly packed column. Autors who discuss the effect of guard columns on the separation efficiency, for precolumns packed with a pellicular sorbent[8] and for CGC columns[9], and on the optimization of the design parameters of the precolumn[10], agreed that the critical factor for the efficiency of the analytical column connected with a precolumn is the geometry of the guard column; it is suggested that the inner diameter of the guard column should be 2–2·5 mm and length 1–2 cm, which correspond to volumes of 30–100 µl. Such a precolumn has no significant effect upon the decrease in efficiency of the analytical column, however, its protective function is rapidly lost and it has to be replaced frequently (every other day in our experiments with biological material); thus, the design according to the above procedure appears to be very convenient. The Teflon precolumn does not suffer damage even if the sealing nuts are maximally tightened (as often do glass columns) or on repeated disassembling and assembling, which is the frequent cause of leaks of stainless steel precolumns with metal-to-metal sealing.

The efficiency of a chromatographic column also depends on the way of sample application. At present in HPLC, loop injectors are used nearly exclusively; in com-

parison to the direct application in the column, this method results in poorer sample introduction. Kirkland and coworkers[8] suggest a highly efficient "point-injection" design, based on the attachment of a secondary inlet capillary to the sealing nut of the column. This capillary is used for the delivery of the pure mobile phase, while the sample is delivered to the packing, or to the screen or frit, through the central ca-

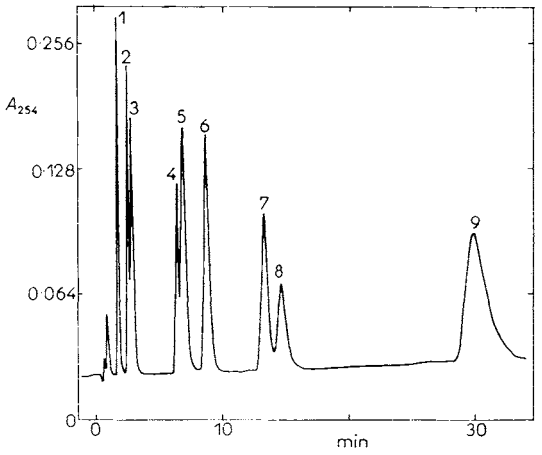

FIG. 3

Chromatogram of a mixture of nucleosides and bases obtained on a CGC column with supplemented packing serving as internal guard precolumn; 254 nm UV detector, gain $32 \cdot 10^{-2}$ AUFS. Mobile phase: 0·1M-$HClO_4$, 10 vol.% ethanol (96%), 0·05% (m/v) sodium dodecylsulphate, pH 2·5; $F_m = 0{\cdot}5$ ml . . min^{-1}. Sample (50 µl) contained: **1** uridine (2·87 nmol), **2** inosine (2·98 nmol), **3** xanthine (1·64 nmol), **4** guanosine (2·47 nmol), **5** hypoxanthine (2·57 nmol), **6** adenosine (1·83 nmol) (1·64 nmol), **7** guanine (6·62 nmol), **8** cytosine (6·75 nmol), **9** adenine (4·44 nmol)

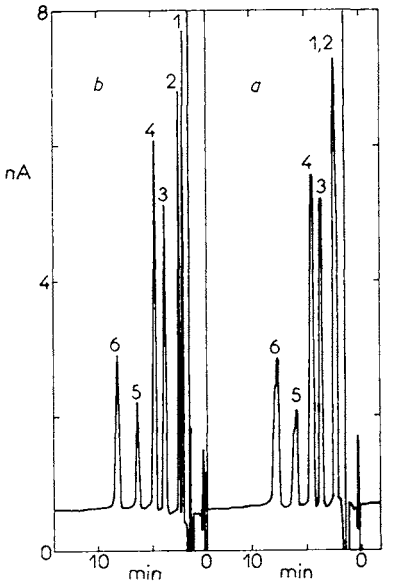

FIG. 4

Chromatograms of standards of monoamines and their metabolites on a CGC column packed with Separon SIX C18 ($d_p = 5$ µm), to which a 3 × 20 mm guard precolumn is linked. (*a*) damaged precolumn packing, (*b*) guard column newly packed with Separon SIX C18 ($d_p = 10$ µm). Electrochemical detector, gain 10 nA/10 mV, 0·5 s filter. Eluent buffer: 0·05M-K_2HPO_4, 0·03% (m/v) Na_2EDTA, 0·03% (m/v) sodium heptanesulphonate, 16 vol.% acetonitrile, pH 3·4; $F_m = 0{\cdot}5$ ml min^{-1}. Sample (5 µl) contained: **1** dihydroxybenzylamine (internal standard) (9·5 pmol), **2** dopamine (7·9 pmol), **3** dihydroxyphenylacetic acid (9·8 pmol), **4** serotonin (6·0 pmol), **5** 5-hydroxyindoleacetic acid (7·8 pmol), **6** homovanillic acid (16·5 pmol)

pillary. This approach has been extended to the intra-column injector concept[11] for stainless steel columns with the central capillary protruding through the screen into the packing, or the radial injection concept[12] with the sample injected radially several centimetres below the top of the packing. The use of the intra-column injector for CGC columns[13] extends their lifetime as much as five times owing to the elimination of pressure pulses from the pump and injector, and it increases the precision of analysis (in terms of relative standard deviation) three times. The use of less efficient old columns with a dead space formed or poorly packed columns is also made possible[11,12]. The effect of the intra-column injector is equal to that shown in Fig. 4 for new and deteriorated guard column. Figs 4*a* and 4*b* demonstrate that the attachment of the injector to a deteriorated column and optimization of the flow ratios from the secondary and central capillaries, usually in the range from 2 : 3 to 7 : 3, results in restoration of the initial column efficiency. The drawbacks of the point injection are that only a fraction of the column bed is employed in separation, and that the inlet capillary can get clogged by the sorbent if sufficient care is not exercised. The injector alone does not protect the column from chemical and/or physical contamination; so, it is more advantageous to attach it to a correspondingly enlarged guard column and thus to combine the protective effects of the two systems on the performance of the analytical column[13].

In some cases, it is more convenient to optimize the separation by the column length adjustment than to vary the elution strength of the mobile phase. Czechoslovak CGC columns are 15 cm long and they can be shortened as desired

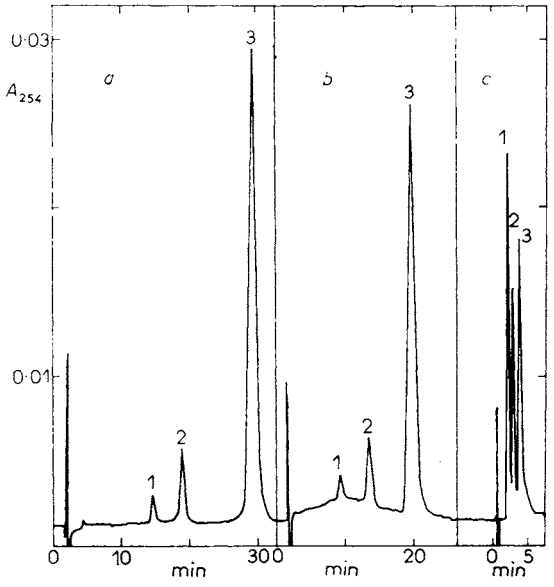

FIG. 5

Chromatograms of adenine nucleotides on a 15 cm (*a*), 10 cm (*b*), and 5 cm (*c*) CGC column packed with Separon SIX C18 (d_p = 5 μm). UV 254 nm detector, gain $4 \cdot 10^{-2}$ AUFS. Eluent: 0·05M-K_2HPO_4, 4 vol.% triethylamine, pH 6·0 adjusted with H_3PO_4; F_m: *a*, *b* 0·5 ml min^{-1}, *c* 1 ml min^{-1}. Sample (20 μl) contained: *a*, *b*: **1** adenosine-5′-monophosphate (0·064 nmol), **2** adenosine-5′-diphosphate (0·315 nmol), **3** adenosine-5′-triphosphate (3·3 nmol); *c*: 15 μl of sample contained compounds **1**—**3**, 1·875 nmol each

without disturbing the chromatographic bed; the efficiency decrease corresponds only to the reduction in the column length, as documented in Fig. 5, showing the separation of adenine nucleotides on a 15 cm column and on the same column shortened to 10 cm and 5 cm lengths. It should be considered, however, that with the 5 cm and 10 cm long columns, extra-column effects play a significant role, which requires adaptations of both the instrument and the detector and the use of an injector suitable for capillary HPLC. In this case, the above conditions were not met, and thus the changes in efficiency with the shortened columns could not be evaluated properly. Still, there is a 10 cm CGC column in use in our laboratory providing successful analyses of ATP, ADP, and AMP in rat brain extracts[14].

As shown in Fig. 1 the outer jacket of the CGC column can be adapted for thermostatting purposes. A water thermostat can be attached to the jacket *via* the nipples, and if the threads of the jacket are sealed with a Teflon tape, temperatures as high as 55°C can be safely applied. Some authors[15,16] reported that increased temperature of the column and mobile phase results in an improved efficiency of resolution on reversed-phase columns, as known in ion-exchange chromatography. Also, a constant temperature is prerequisite for accurate measurements of the retention characteristics.

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