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MICRO-COLUMN LIQUID CHROMATOGRAPHY WITH MULTI-WAVELENGTH PHOTOMETRIC DETECTION

I. THE OB-4 MICRO-COLUMN LIQUID CHROMATOGRAPH

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SUMMARY

The OB-4 micro-column liquid chromatograph is designed for high-performance liquid chromatography (HPLC) on 50–200 μl columns with typical eluent volumes of 500–2500 μl. The detector is a double-beam microspectrophotometer with two flat-window flow cells. The monochromator of the detector contains a special drive for rapid change of wavelength according to stepwise cyclic programmes over the range 190–360 nm. In this way multi-wavelength detection on a time-share basis is performed. The OB-4 chromatograph may be used for all types of HPLC. It consumes much smaller amounts of eluents, adsorbents and samples than other instruments. The multi-wavelength facility helps in identification and evaluation of homogeneity. Examples of applications are given.

INTRODUCTION

In 1969 Kuzmin *et al.*[1] published procedures for micro-column liquid chromatography with photometric detection. They performed both isochratic and gradient elution on 0.5–1.0 μl columns with typical eluent volumes of 1–20 μl. The columns were made of UV-transparent glass. The parts below the filter were used as photometric cells with efficient volumes down to 0.01 μl. Detection was performed by means of a specially designed double-beam microspectrophotometer. Much later, similar techniques were described by other workers[2].

Use of a cylindrical, laterally illuminated cell of an optically imperfect shape makes it difficult to perform spectral measurements. The results are strongly affected

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by changes in the refractive index. However, the major disadvantage of the techniques described above[1] was its unusually small scale, inconvenient for routinous analytical work. The techniques depended on microscopic control.

Nevertheless, outstanding advantages of the micro-column techniques[1] also became obvious. These were (i) an increase in the sensitivity of the analysis (10^{-9} g of nucleotides could be detected); (ii) great saving in adsorbents and eluents; (iii) high speed of the analysis. The advantages of micro-column chromatography stimulated developments aimed at its adaption to routine laboratory practice.

In order to approach to this goal, a new detector was designed. This was a double-beam microspectrophotometer with a 2-μl flat-window flow cell[3,4]. Appropriate micro-column chromatographic techniques have been also elaborated[5,6]. The typical volumes of ion-exchange chromatographic columns were increased to 50 μl, and typical volumes of the eluent to 600 μl. These techniques became popular in some fields of analysis[6–9]. An important advantage of the new detector was the possibility of multi-wavelength detection on a time-share basis. However, the change of the wavelength was slow, one measurement of the absorbance at a single wavelength taking 30 sec.

In subsequent years, our group continued developments, the aim of which was to design an instrument and methods for micro-column high-performance liquid chromatography (HPLC). The following tasks were to be solved: (i) extension of the dynamic range, increase in precision and sensitivity and decrease in the time of response of the detector; (ii) development of a complete micro-column liquid chromatograph, including detector, pump, sampler, columns and fraction collector, suitable for HPLC on 200-μl columns with typical eluent volumes of 1000–2000 μl; (iii) development of procedures for micro-column HPLC with multi-wavelength detection of compounds of different classes. The outcome of these developments is the OB-4 micro-column liquid chromatograph. The design of this instrument and some applications are described below.

DESIGN OF THE OB-4 CHROMATOGRAPH

General features

Fig. 1 shows the general view of the OB-4 chromatograph. The instrument consists of the following main units: (1) electronic unit; (2) optical unit; (3) pump; (4) sampler and column and (5) fraction collector.

The functions of the instrument are operated mainly from the front panel of the electronic unit. The right-hand part of the panel serves to operate the detector. Using keyboard 6, one may select a cyclic programme of up to eighteen wavelengths, from 190 to 360 nm, at 10-nm intervals. Alternatively, one may choose the single-wavelength mode and set any wavelength within the same range, at 2-nm intervals. One may also scan the UV spectrum at 2-nm intervals. By means of switch 7, the time of the measurement (integration) at given wavelength(s) may be chosen within the interval from 0.15 to 20 sec.

The instrument is calibrated as if the absorbance was measured in a 1-cm cell (in a.u.f.s.), although the actual length of the cell is smaller. The range of the instrument is from -0.96 to $+12.8$ a.u.f.s. The right-hand part of the digital display shows the wavelength and the middle part shows the absorbance value. The full scale of

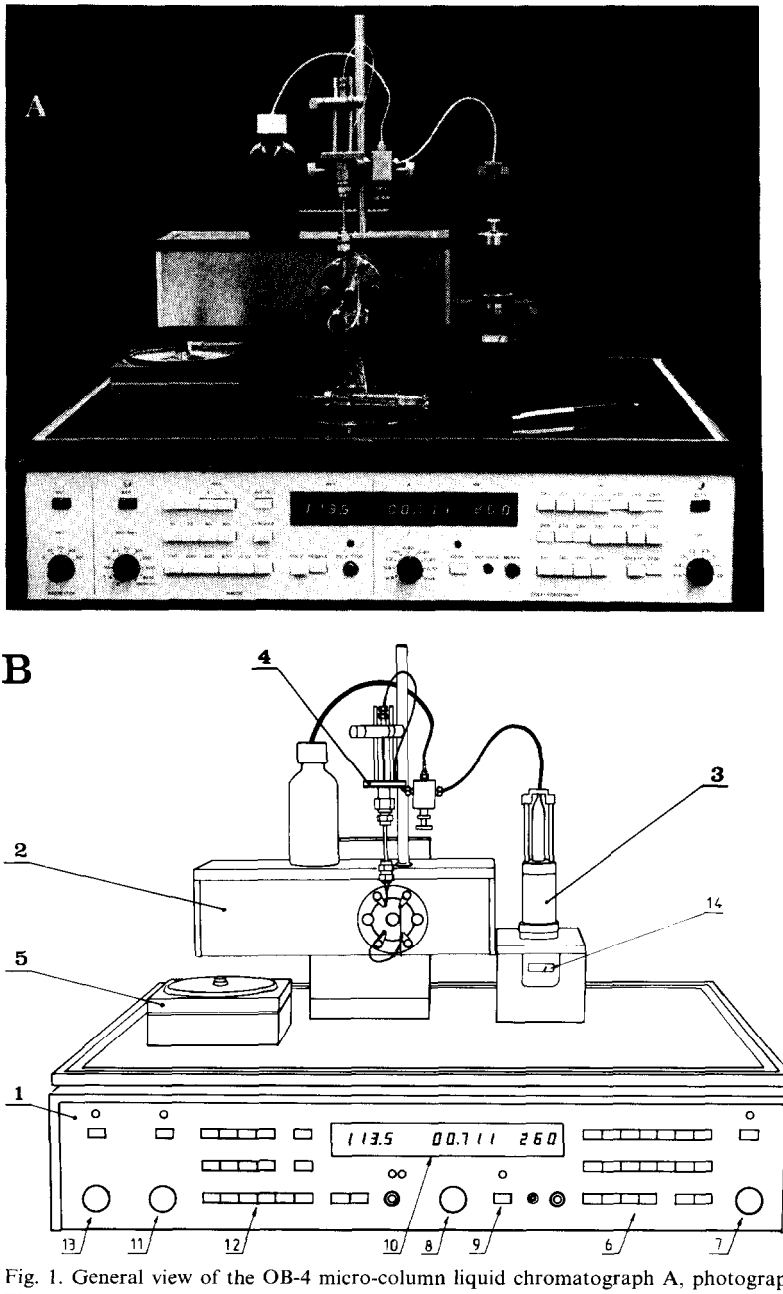

Fig. 1. General view of the OB-4 micro-column liquid chromatograph A, photograph; B, scheme. 1 = Electronic unit; 2 = optical unit; 3 = pump; 4 = sampler and column; 5 = fraction collector; 6 = keyboard of monochromator; 7 = integration time switch; 8 = scale switch; 9 = fold scale switch; 10 = digital display: left-hand part, microlitres delivered or taken by pump; middle part, absorbance as if measured in a 1 cm cell; right-hand part, wavelength (nm); 11 = flow-rate switch; 12 = keyboard of pump; 13 = fraction volume switch; 14 = pressure indicator.

absorbance presented on the recorder may be selected by switch 8; it may be equal to 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 or 12.8 a.u.f.s. Switch 9 serves for "scale folding" (see below).

The left-hand part of the electronic unit front panel operates the liquid circuitry of the chromatograph. By means of switch 11 one may select the rate of eluent delivery at 600, 200, 100, 50, 30, 20, 15, 10, 5 or 2 μl/min. One of the positions corresponds to rapid flushing of the pump. Keyboard 12 serves to select the direction of pump piston movement, and the volume after the delivery (or intake) of which the pump will stop. This volume may be selected within the range from 0.1 to 2500 μl. The volume delivered or taken is shown in the left-hand part of the digital display 10. Switch 13 serves to select the volume of fractions collected by the fraction collector within the range from 5 to 50 μl.

Pump 3 contains a pressure indicator (14). The pump stops and produces an audible signal after its piston reaches the highest or the lowest position, and when the pressure exceeds 5 MPa.

The upper lid of the electronic unit is made of a chemically inert polymer. The dimensions of the chromatograph are 70 × 50 × 30 cm and the weight is 42 kg. Presented below are the most important details of the design.

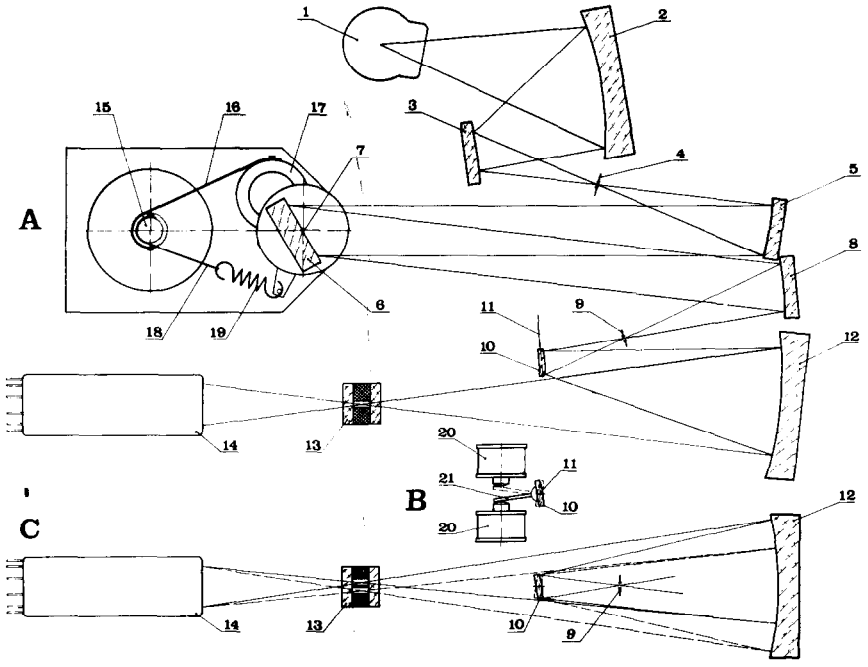

Fig. 2. Optical and mechanical diagram of the detector of the OB-4. A, Top view; B, electromagnetic drive of the convex oscillating mirror; C, the double-beam photometric scheme, side view. 1 = Deuterium lamp; 2 = concave mirror; 3 = cylindrical convex mirror; 4 = slit; 5 = concave mirror; 6 = diffraction grating; 7 = axis of rotation of the grating; 8 = concave mirror; 9 = diaphragm; 10 = convex oscillating mirror; 11 = axis of rotation of the convex oscillating mirror; 12 = concave mirror; 13 = cells; 14 = photomultiplier; 15 = shaft of the stepping motor; 16 = steel band; 17 = excentric ring; 17 = nylon thread; 19 = spring.

Detector

The optical diagram of the double-beam microspectrophotometer which comprises the detector of the OB-4 is shown in Fig. 2. The monochromator is built on the basis of a flat holographic grating (3600 lines mm^{-1}). To change the wavelength, the grating is turned by a stepper-motor via a sine mechanism consisting of a flexible steel band and a ring fixed excentrically on grating shaft[10]. The double-beam photometric part is a mirror objective whose convex mirror is oscillating and may be fixed by an electromagnetic drive in two different positions in order to focus light either on the reference or the sample cell[3,4].

The cell unit (Fig. 3) is a polyfluoroethylene gasket with two holes, one of which is the reference and the other the sample cell. The gasket is pressed between two flat silica windows. Eluent (or reference solvent) is delivered into the cells through 0.1 mm I.D. polyfluoroethylene capillary tubes.

The electronic measuring part of the microspectrophotometer is a logarithmic, integrating digital voltmeter, the principle of action of which is based on the exponential discharge of a capacitor. One measurement cycle takes 160 msec (8 mains cycles). The light flux of the reference channel is transformed into electric current by a photomultiplier. This current is integrated during 40 msec by means of an integrator consisting of a capacitor and a high-gain amplifier. After the integrator has been disconnected from the photomultiplier, the capacitor is short-circuited by a resistor, and the time of discharge to a given potential is measured digitally and stored in a register. After this, the light beam is directed to the sample cell, the current of the sample channel is integrated and the capacitor is discharged through the same resistor to the same potential. The time of the discharge is now subtracted from that stored in the register. The difference remaining in the register is proportional to the logarithm of the ratio of the reference and the sample photocurrents, and therefore to the absorbance.

The value of this difference is multiplied by an arbitrary calibration coefficient in order to obtain the result in a.u.f.s. units, and the latter is shown on the digital display. Via a digital-to-analogue convertor, the same value is also fed into the re-

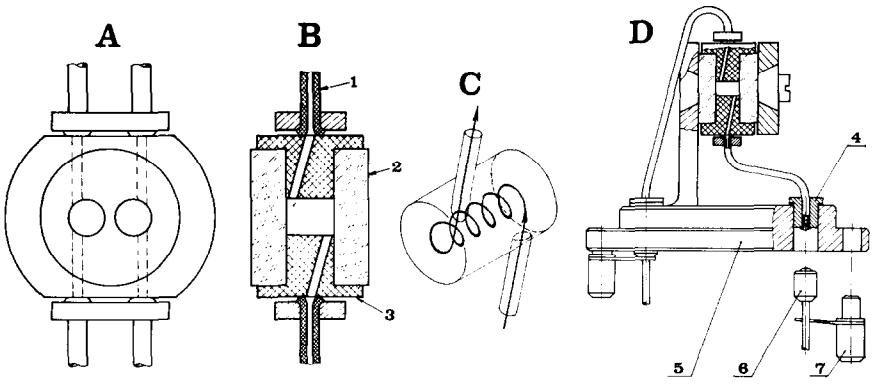

Fig. 3. Cell unit. A, Front view; B, side view; C, scheme of cell hydrodynamics; D, cell unit in holder, side view. 1 = Inlet tube; 2 = silica window; 3 = polyfluoroethylene gasket; 4 = immobile part of the liquid connector; 5 = body of the holder; 6 = mobile part of the liquid connector; 7 = spring of the liquid connector with handle.

corder. Besides the above-mentioned actions, the electronic unit performs autocalibration of the voltage on the photomultiplier according to the light flux of the reference path, as well as autocalibration of the characteristics of the elements of the measuring circuitry, during each measurement cycle.

In order to increase the signal-to-noise ratio, the measurement cycles may be repeated; the number of measurements may be selected by the switch 7 (Fig. 1); averaging of the results is performed digitally.

Pump

The pump is of the displacement (syringe) type. The total displaced volume is 2500 μl. The cylinder of the pump is a precision-bore 9 mm I.D., 14 mm O.D. borosilicate glass tube. The piston tip is made of tantalum. The polyfluoroethylene O-ring of the piston is constantly forced to the wall of the cylinder by means of a cone and a strong spring. The piston is moved by means of a clearence-less screw-and-gear mechanism activated by a stepping motor. The pump contains a pressure sensor which measures the force between the thrust face of the driving screw and the piston. The signal from the sensor is fed via an amplifier into an indicating galvanometer. The pressure limit of the pump is adjusted to 5 MPa.

Columns and sampling

In order to meet the requirements imposed by different chromatographic procedures, the chromatograph is equipped with columns of two different types. The columns for ion-exchange chromatography in highly aggressive solvents are made of 0.8–1.0 mm I.D. glass tubes of length 20–50 mm, the lower part of which above the nozzle contains a fritted glass filter, and the upper part is formed as a cone. The column is put into a holder (Fig. 4A) by means of which a polyethylene tube is pressed to the cone; the end of the polyethylene tube is formed as a hemisphere by holding it in a flame. The outlet tube of the pump in this instance is equipped with a glass capillary tube with fire-polished tips, which fits tightly the polyethylene tube of the column. This type of connection holds pressures up to 1.5 MPa; it may be used with extremely corrosive eluents, *e.g.*, concentrated hydrochloric acid.

The columns of the second type are made of stainless steel (Fig. 4B) and are used for HPLC in less aggressive solvents. In this instance, the outlet tube of the pump is connected to a two-way valve (Fig. 4C) which, in turn, is connected to the sampler needle and the eluent reservoir. The sampler is of the stop-flow type. To take a sample, the valve is closed and the sampler needle is removed from its nest and dipped into the sample solution. After this, a sample of desired volume is sucked into the needle by moving the pump piston down to a given distance according to volume selected by means of the keyboard 12 (Fig. 1). The needle is then inserted into its nest on the sampler and tightened by means of the screw which compresses the polyfluoroethylene O-ring. After the tightening, application of the sample and elution proceed uninterruptedly.

The columns are precision-bore stainless-steel tubes with polished inner walls of 2 mm I.D. Both ends of the column are covered by stainless-steel frits (2 μm pores, 0.1 mm thickness). The length of the column is 62 mm and the volume is 200 μl.

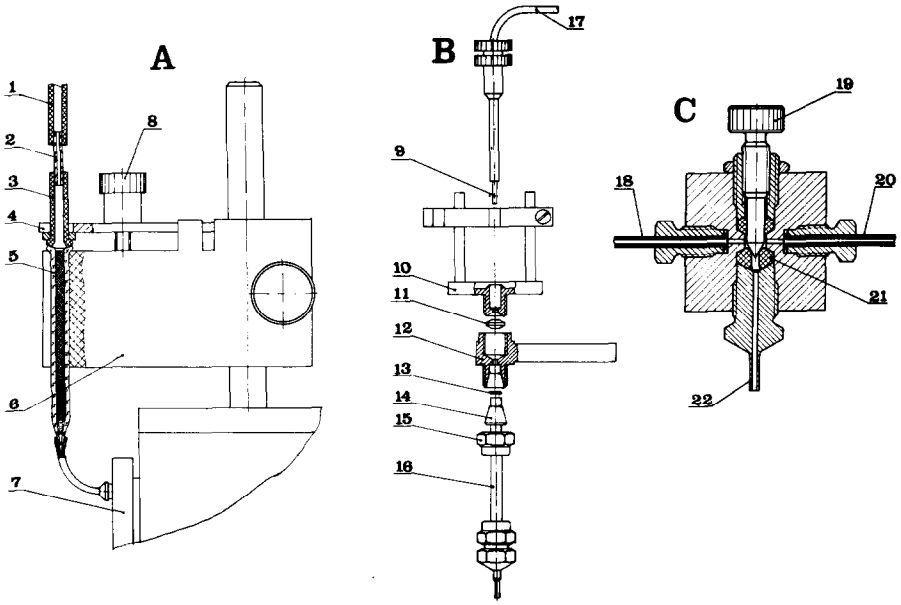

Fig. 4. Columns, holders, sampler and valve. A, Glass column in holder; B, stainless-steel column in holder-sampler; C, valve. 1 = Polyethylene or polyfluoroethylene outlet tube of the pump; 2 = glass capillary tube; 3 = polyethylene tube with its lower end heated in a flame to form a hemisphere; 4 = spring; 5 = glass column; 6 = holder; 7 = body of the cell holder; 8 = screw; 9 = 0.2 mm I.D. stainless-steel tube; 10 = sealing screw; 11 = polyfluoroethylene O-ring; 12 = holder; 13 = frit; 14 = cone; 15 = nut; 16 = body of column; 17 = tube, to valve; 18 = tube, to column; 19 = screw; 20 = tube, to pump; 21 = polyfluoroethylene O-ring; 22 = to eluent reservoir.

Fraction collector

The eluate flowing out of the detector cell may be collected as fractions of equal volume, the size of which is selected using the switch 13 (Fig. 1). This task is performed by the fraction collector 5 (Fig. 1), which contains a polyfluoroethylene disc with 100 craters serving as "test-tubes". At each step, the electromagnetic drive of the collector forces the disc down and rotates to 1/100 of a full turn. The capillary tube which delivers the eluate touches the bottom of the crater (Fig. 5). Because of this it is possible to collect fractions the volumes of which are smaller than that of free droplets, *e.g.*, 5 μl.

SUBSTANTIATION OF THE DESIGN AND PERFORMANCE

It is obvious that, within fairly wide limits, proportional miniaturization of the column, cell, eluent and sample volumes and adsorbent particle diameter may not change the result of chromatography. This has been confirmed by Kuzmin *et al.*[1], who miniaturized the scale of chromatography about 10,000 times compared with that conventionally used at the time. The advantages of miniaturization have been outlined earlier[11]. The first and the major advantage of miniaturization is the increase in the sensitivity of the method which follows from the fact that the same amount of substance will be eluted from a micro-column at a higher concentration than from a normal-size column, and hence the "chemical" signal-to-noise ratio becomes greater.

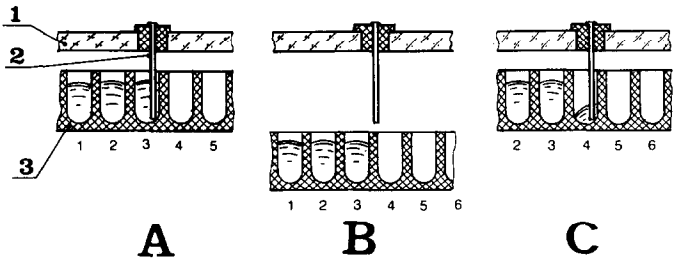

Fig. 5. Scheme of action of the fraction collector. A, End of collection of fraction No. 3; B, electromagnetic drive pulled the disc down and rotated it to 1/200 of full revolution; C, start of collection of fraction No. 4; the disc was lifted by the drive and rotated to 1/200 of full revolution. 1 = Glass cover; 2 = polypropylene capillary tube; 3 = PTFE disc with craters. Small numbers: fraction numbers.

Secondly, the consumptions of solvents and adsorbents become smaller. In addition, miniaturization leads to a decrease in dimensions and to simplification of the equipment.

At the beginning of the development of the OB-4 instrument, it was necessary to choose the scale of miniaturization. This choice was made on the basis of the following speculations. The existing chromatographs make it possible to run typical high-performance separations on 2–5 ml columns with 5–20 ml of eluent, and typical columns have total efficiencies of 3000–10,000 theoretical plates. However, the now available 3–5 μm adsorbents may be packed into columns of such a total efficiency having volumes 200–500 μl. If this scale is taken as the basis, typical volumes of the eluents will be 0.5–2 ml. The variances (half-widths at 0.607 of the peak height) of the narrowest peaks will be greater than 2 μl, and the maximum volumes of sample may be 0.5–1 μl[12,13].

The dimensions of the detector cell had to be optimized for both hydrodynamic and optical performance. Substantiation of the cell dimensions is considered in the following section.

Performance of the detector

The detector plays a major role in the performance of the chromatograph, and therefore the factors that affect its characteristics will be considered in more detail. Conditions for maximum precision of photometric measurements by means of double-beam spectrophotometers have been discussed earlier[4]. These are, by definition, the conditions under which the noise, the drift and the shift of the baseline due to changes in wavelength are minimal, and the response to absorbance is linear.

Long-term drift of the baseline in double-beam spectrophotometry depends on the instability of the position of the luminous body of the light source, and on the change in the sensitivity of different parts of the photocathode. In order to bring the drift to a minimum, the optical diagram must meet the following requirements: (a) the same light beam must strike alternately the reference and the sample cell; (b) the light beam must not touch the side walls of the cell; (c) the light beam leaving the reference and sample cells must strike the same area of the photocathode.

If these requirements are met, changes in the geometry and intensity of the light beam striking the cells and changes in the sensitivity of the photocathode will result in

identical changes of the reference and sample signals, and thus will be compensated for by the electronic measuring unit.

The shift of the baseline due to changes in the wavelength is a consequence of the different spectral characteristics of the optical elements which are not common to the reference and the sample light paths. Hence, in order to bring the shift of the baseline to a minimum, it is necessary to minimize the number of elements that are not common to the reference and the sample paths.

All of these requirements are met by the double-beam microspectrophotometer which is the detector of the OB-4. It can be seen in Fig. 2 that the mirror objective (mirrors 10 and 12) used as the light switch directs the same light flux on to the reference and the sample cells, and that switching of the channels does not lead to a shift of the light spot on the photocathode. The objective focuses light on the cells in such a way that it does not touch the side walls. The optical elements which are not common to the reference and the sample paths are only the cells themselves. Switching of the light beam from the reference to the sample cell results in only a small shift of the light spot on the surface of the concave mirror 12 (Fig. 2) of the objective, and different parts of this mirror do not have considerably different reflectance spectra. Therefore, the spectral shift of the zero line is small.

In order to choose optimal dimensions of the photometric cell, one has to consider that an increase in the path length of a cell of given constant volume results in a decrease in the signal-to-noise ratio in absorbance measurement. This happens because the light flux that can be passed through a cell decreases with decrease in the cell diameter, and hence the noise, which depends on the light flux, increases more rapidly than the signal, which depends on light path. Therefore, in order to achieve the highest signal-to-noise ratio with a cell of given volume one has to reduce the light path, rather than increase it, as prompted by intuition.

Cells with short light paths are also advantageous because they give the possibility of obtaining a wider dynamic range of linear response, of a photometer to substance concentration, as precise measurement of small absorbances is much simpler than linearity of the response at high absorbances.

However, the cell must be optimized for both hydrodynamic and optical characteristics. Obviously, narrow and long cells have better hydrodynamic characteristics. The theory of the flow of eluates through photometric cells[12,13], unfortunately, is based on the assumption that all the cross-section of the cell is illuminated, and for this reason could not be directly applied to the cells of the OB-4 instrument.

The dimensions and the design of the cells of the OB-4 have been chosen empirically, by direct comparison of the characteristics of cell prototypes. The best results were obtained with cells of the Z-type, and with tangential delivery of the eluate (Fig. 3). The delivery and the exit capillary tubes have I.D. 0.1 mm, and their length is 25 mm. The diameter of the cell is 1.2 mm and the optical path length is 1.6 mm. In order to check the dynamic characteristics, an "infinitely small" volume (0.1 μl) of an absorbing solution was sucked into the cell pre-filled with solvent, and the cell was washed (by suction) with the solvent at different flow-rates. The results obtained are shown in Fig. 6. It should be mentioned that these results were obtained with an assembled cell, together with the inlet and the outlet capillary tubes.

Specific requirements must be met by the monochromator of the spectrophotometer intended for monitoring eluates at several wavelengths on a time-share basis.

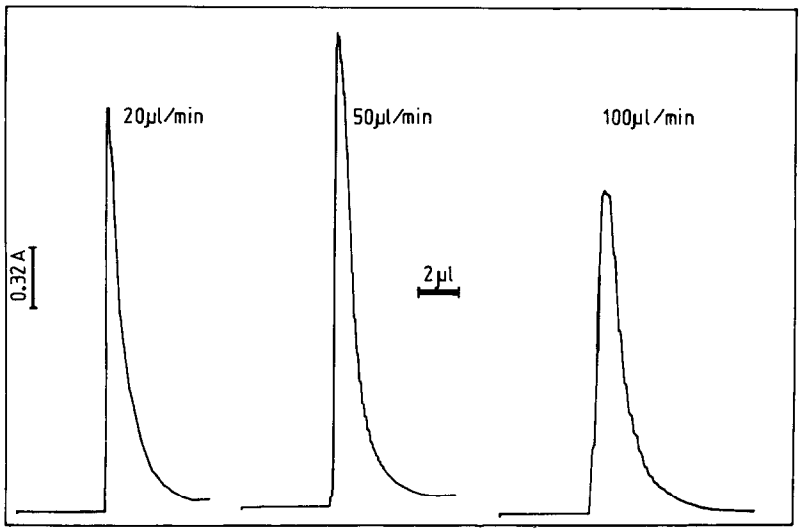

Fig. 6. Test for the cell dynamic volume. The cell was filled with methanol. After this, the nest of the liquid connector (Fig. 3D) was filled by a solution of *p*-nitroaniline in methanol, and 0.1 μl of this solution sucked by pump into the cell inlet tube. The nest of the connector was thoroughly washed and filled with methanol, and the pump switched on for suction. At the same time, the recorder drive was also switched on at a rate proportional to that of the pump. The absorbance (A_{360}) results monitoring at three flow-rates are shown.

First, the monochromator has to give the greatest possible light flux in order to provide a high signal-to-noise ratio in rapid detection. To meet this requirement, one has to look for a compromise with the spectral resolution. The spectral slit half-width in the OB-4 is 5 nm. This wide slit is suitable for most substances studied by HPLC, because the UV spectra of solutions usually do not exhibit fine structure. In necessary cases, *e.g.*, with aromatic hydrocarbons that do exhibit fine structure, in order to compare the spectral data with the published information, one has to reduce the published spectra to 5 nm resolution by calculation.

Similar speculations were the basis for the selection of the 2 nm step in the spectrum scanning mode, and of the 10 nm step in multi-wavelength detection. Use of smaller steps or of continuous scanning typical of other detectors gives unnecessarily excessive information and considerably complicates the design.

Next, it was necessary to select the wavelength range, which in the OB-4 is 190–360 nm. The shorter wavelengths of this range are very useful for the detection of many non-aromatic compounds. On the other hand, the absence of the visible part of the spectrum does not reduce the scope of the detector to any considerable extent, because all the compounds that absorb visible light also absorb UV light. Extension of the spectral range of the detector to the visible region would result in an increase in its dimensions and complicate the design.

Of greatest importance in multi-wavelength detection is the rapidity of wavelength change and the accuracy and repeatability of its setting. The time during which a peak is eluted in HPLC may be as short as a few seconds. Therefore, the monochromator must change the wavelength rapidly. In order not to spoil the chromatographic resolution, the cyclic programme of, *e.g.*, three wavelengths must be repeated 7–10

times over the peak, *i.e.*, one measurement of the absorbance at a single wavelength must be made during a few tenths of a second.

A high accuracy of the wavelength setting is necessary to compare the spectral information obtained in multi-wavelength detection with the published UV spectra. Even greater are requirements for the repeatability of the wavelength setting, because insufficient repeatability would result in multi-wavelength detection in additional noise at wavelengths corresponding to steep slopes of the UV spectra of eluates.

The requirements outlined above are met by the design of the monochromator of the OB-4. The key to the problems is the above-mentioned sine mechanism shown in Fig. 2. With this low-inertia mechanism, a single measurement at any wavelength of a cyclic programme takes no more than 0.3 sec. The accuracy of the wavelength setting as checked by measuring the UV spectrum of chromate ion[14] is ± 0.5 nm. Repeatability of the wavelength setting, as evaluated by repeatability of the absorbance measurement in the multi-wavelength mode in the region of a steep slope of the UV spectrum, is better than ± 0.01 nm.

Performance of the detector of the OB-4 is illustrated by Fig. 7. In addition, it should be mentioned that no drift of the baseline occurred even over very long periods with a cell imitator (a plate with two holes of the same form as the cells). As for the drift with the real cell, it always had a "chemical" nature and was due to a change in the composition of the solution inside the cell.

Characterization of the detector of the OB-4 as a spectrophotometer, an instrument for absolute measurement of absorbance, is a complicated task that has not been completely solved so far because of the absence of appropriate standards. However, it is obvious that linearity of the response over a wide range of concentrations rather than the absolute accuracy of the absorbance measurements is of greatest importance in liquid chromatographic detection. Linearity of the response over the

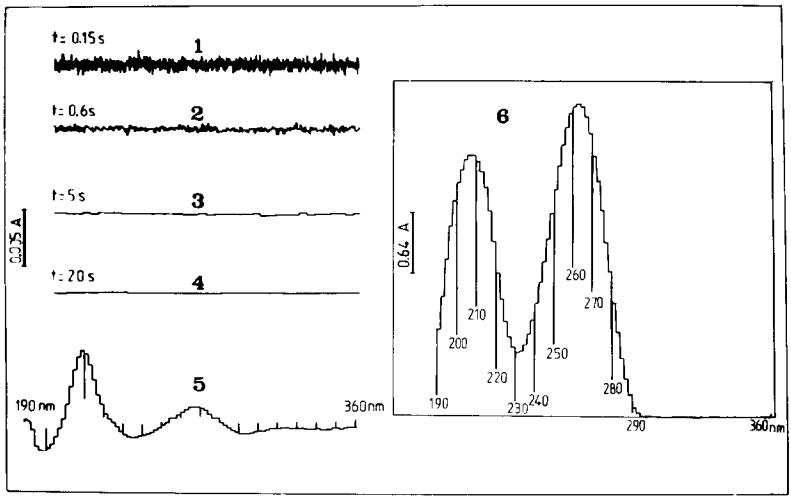

Fig. 7. Some characteristics of the detector of the OB-4: 1–4, noise at 270 nm and integration times (1) 0.15 sec; (2) 0.6 sec, (3) 5 sec, (4) 20 sec; 5, spectral baseline with cell imitator; 6, UV spectrum of an aqueous solution of uridine measured during 102 sec.

dynamic range is important for correct measurement of the areas of chromatographic peaks relative to standards and, especially, for correct calculation of the absorbance ratio.

The linearity of the response of a spectrophotometer to absorbance is determined by three factors: first, by the quality of the monochromator and by the ratio of the useful and the stray light fluxes; second, by the linearity of the response of the photomultiplier; and third, by the quality of the treatment of signals by the electronic unit, *i.e.*, by the precision with which it converts the photocurrents of the reference and the sample channels into the logarithm of the ratio of these photocurrents.

The last of the three tasks is solved in the OB-4 with high precision. Special electrical measurements revealed that the linearity of the response of the electronic unit to the logarithm of the ratio of two reference currents has a precision better than $\pm 0.1\%$ over the range of absorbances from 0 to 6.4 a.u.f.s., and a precision better than $\pm 0.5\%$ over the range from 6.4 to 12.8 a.u.f.s.; these values correspond to a logarithm of the photocurrent ratio between 0 and 2 (as mentioned above, the instrument presents the absorbance values as if measured in 1 cm light path cells, although the actual path is smaller, 0.16 cm).

Linearity of the response of the detector to absorbance was checked using solutions. In one of the experiments, potassium chromate solution[14] was taken. A concentrated solution of this compound was diluted gravimetrically, and the absorbance of the diluted solutions obtained was measured with the detector of the OB-4. The results are shown in Fig. 8. It can be seen that the linearity over the range of absorbances from 0 to 10 is better than $\pm 1\%$.

It has been mentioned above that linearity of the response to absorbance in multi-wavelength detection is important for correct measurement of the absorbance ratio. In this connection, one more experiment was made, in which a concentrated

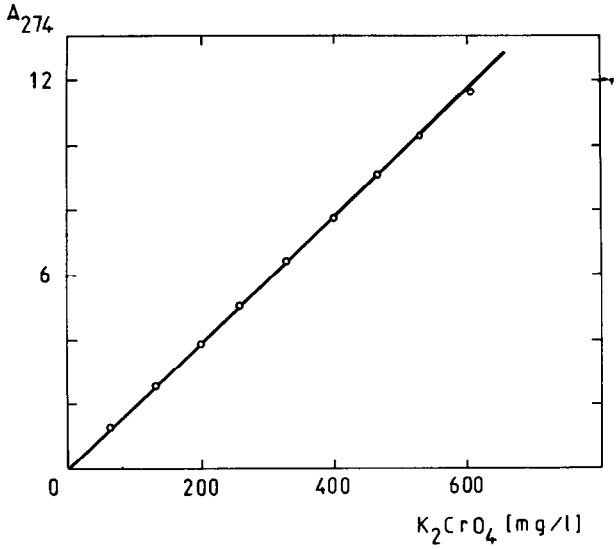

Fig. 8. Dependence of the absorbance at 274 nm displayed by the detector of the OB-4 on the concentration of potassium chromate.

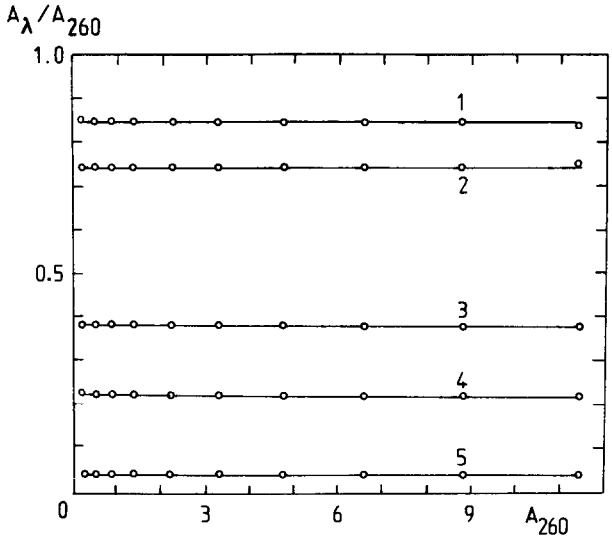

Fig. 9. Dependence of the A_λ/A_{260} absorbance ratio on the absorbance at 260 nm of an aqueous solution of uridine as measured with the detector of the OB-4. 1, A_{270}/A_{260}; 2, A_{250}/A_{260}; 3, A_{280}/A_{260}; 4, A_{230}/A_{260}; 5, A_{290}/A_{260}.

solution of uridine was diluted several times and passed through the cell. The absorbance ratio A_λ/A_{260} was calculated at several dilutions. The results are shown in Fig. 9. The linearity is better than $\pm 1\%$.

Unfortunately, solutions are not suitable for determination of the performance of the instrument at a higher accuracy. The sources of errors with solutions are possible deviations from Beer's law at high concentrations of solutes, the presence of dust particles, the possibility of changes due to ageing, adsorption of solutes on cell walls, etc. More comprehensive studies of the linearity will be made later.

As for the absolute accuracy of the measurement of absorbance, it is, besides the above linearity and the light monochromaticity, determined also by the accuracy of the machining of the cell, by the actual light path length. This accuracy is not high ($\pm 5\%$), but it is not of great importance in liquid chromatographic applications.

Performance of the pump

Besides the above-discussed characteristics of the detector, the quality of the pump is of no less importance for the performance of a chromatograph. At present, most commonly used in HPLC are reciprocating pumps. In order to remove pulsations, such pumps are equipped with special mechanical and electronic devices. An advantage of reciprocating pumps is the simplicity of changing the eluents, and their "constant readiness" in serial analyses.

However, it is not a simple task to design a reciprocating pump for micro-column chromatography. The main difficulty is the design and manufacture of precise miniature and corrosion-resistant valves. Because of these difficulties, we selected the displacement principle, according to which the pump piston moves in one direction during all given chromatographic experiments. The displaced volume of the pump is small (2500 μl), and the pressure necessary in micro-column liquid chromatography is

moderate (5 MPa). Therefore, the cylinder of the pump may be made of glass. As a result, cleaning of the pump and removal of air bubbles may be performed under visual control. Moreover, the glass cylinder makes the pump extremely corrosion-resistant. The precision-bore tube (9 mm ± 10 μm) is inexpensive.

The drive of the pump is a high-precision stepping motor (200 steps for a full turn). The angle corresponding to one step is held within an accuracy of ±3%. One step of the motor corresponds to a displaced volume of 0.05 μl. The high precision of the drive makes it possible to use the pump for not only the delivery of the eluent, but also for the application of sample. The mean square error of the application of a 2 μl sample is ±2%, as shown by chromatographic experiments (Fig. 10). The accuracy of the sampling was evaluated by displacing and weighing nominal 100-μl volumes of water; the error was ±0.3%. For rapid change of the solvent, the head of the pump is made easily detachable from the drive; washing may be effected manually.

Using the same single pump, it is possible to perform not only isochratic, but also gradient elution. This possibility is based on the fact that the specific gravities of gradient components are always different. The gradient is prepared and stored within the pump cylinder. For example, let us assume that one is going to use a linear gradient of sodium chloride from 0 to 0.3 *M* with a total volume 2200 μl. In this instance, the starting 0 and 0.3 *M* sodium chloride solutions are taken, and nine more solutions are prepared by mixing the stock solutions in the proportions 9:1, 8:2, ..., 1:9. The pump is pre-washed with 0.3 *M* sodium chloride, and the piston is driven into a position of complete displacement. After this, 200 μl of each of the solutions prepared are taken in the sequence 0.3, 0.27, 0.24, ..., 0 *M*. Owing to mixing in the

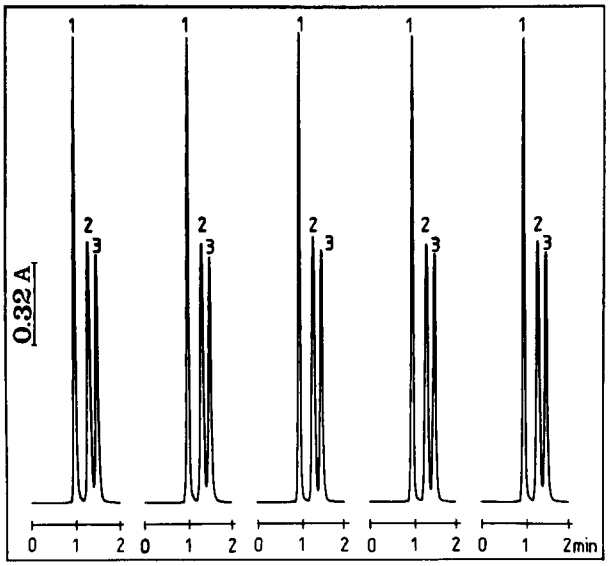

Fig. 10. Illustration of the repeatability of sampling. Five repeated separations of a mixture of three nitroanilines (1 = *ortho*-; 2 = *meta*-; 3 = *para*-). Sample: 2 μl (2 μg of each of the compounds). Column: 62 × 2 mm I.D. Silasorb 600 (5-μm particles). Eluent: chloroform. Flow-rate: 200 μl/min. Wavelength: 290 nm.

inlet tube, a smooth rather than a stepwise gradient is formed, the shape of which is approximately linear. After the formation of the gradient, the sample is taken, the pump connected with the column, and, uninterruptedly, application of sample and elution is started.

The small difference in the specific gravities of sodium chloride solutions of different concentrations is sufficient to stabilize the gradient against convection. As for the diffusion, it is slow, and experiments with coloured solutions showed that the gradient stored within the pump tube is stable for hours.

In this way it is possible to make not only linear gradients, but also monotonous gradients of any shape, and even multi-component gradients, taking into account the specific gravity difference.

If the specific gravity of the solvent mixture directed to the column in gradient elution should decrease rather than increase (this is the case, *e.g.*, in reversed-phase chromatography), the pump is fixed upside-down on a special stand.

Thorough testing revealed that the method of gradient elution described gives reproducible results (Fig. 11). It should be mentioned that the same principle of a "stored gradient" has long been used in centrifugation. Obviously, the use of this method simplifies the equipment. One more advantage is the fact that it overcomes the problem of the decrease in the volume of liquids caused by their mixing.

EXAMPLES OF CHROMATOGRAPHIC SEPARATIONS

Chromatography in aqueous solutions at high column selectivities

It has been mentioned above that using the glass-column version (Fig. 4A) it is

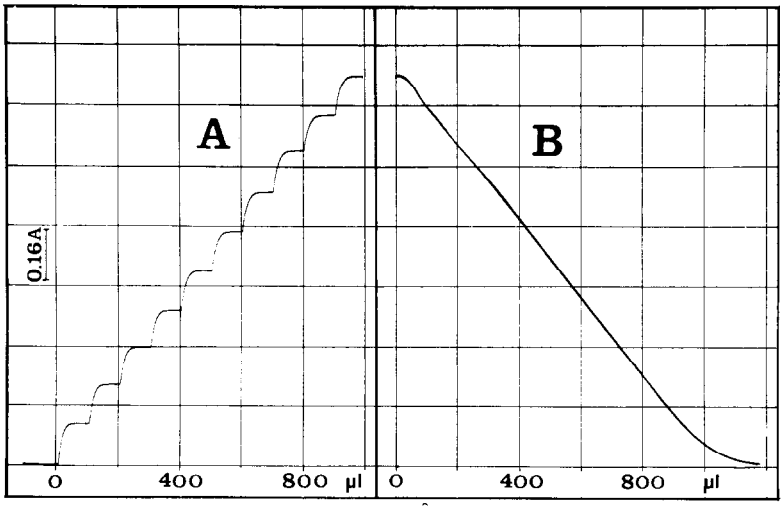

Fig. 11. Repeatability of gradient formation. Two stock solutions were prepared: A, aqueous solution of uridine; B, 60% (v/v) aqueous methanol. Solutions A and B were mixed in separate vessels in the proportions 9:1, 8:2, ..., 1:9. The pump was filled with solution A, and 2000 µl were displaced. After this, 100 µl of each of the solutions were sucked through the cell into the pump in a sequence corresponding to a monotoneous increase in the concentration of methanol. The flow-rate was 200 µl/min. The record of the absorbance at 260 nm is shown in (A). In 5 min, the contents of the pump were forced through the cell at the same flow-rate (B). In (B), the records of two experiments overlap.

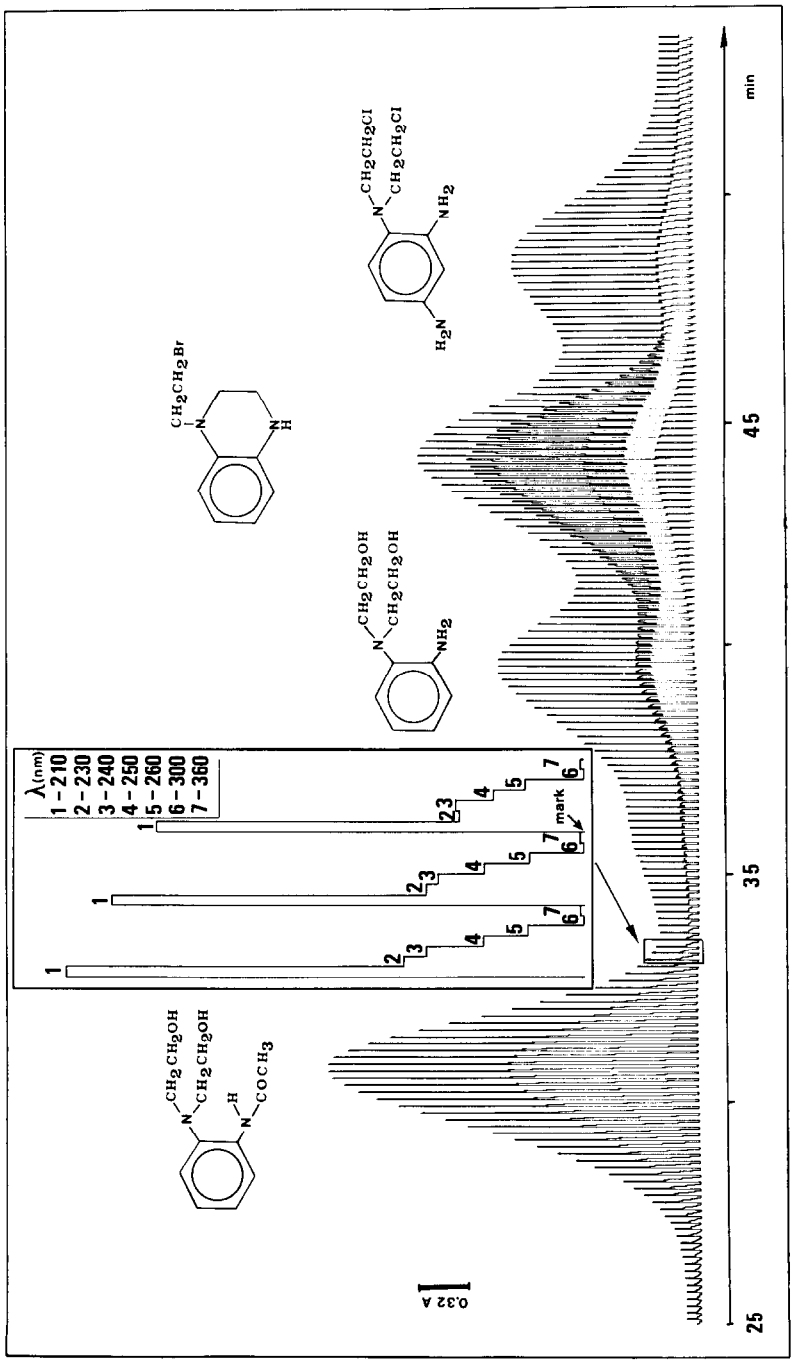

Fig. 12. Separation of aromatic amines on Dowex 50-X8. Column: 70 × 1 mm I.D. Linear gradient of HCl (0.4–4 *M*) in 50% ethanol, 1200 μl; flow-rate, 20 μl/min. Sample: *ca.* 5 μg of each of the amines. Multi-wavelength detection at 210, 230, 240, 250, 260, 300 and 360 nm. Time of measurement at each of the wavelengths: 1.2 sec. The insert is an enlarged part of the chromatogram which illustrates the time-share principle. The method was elaborated to make kinetic studies[15].

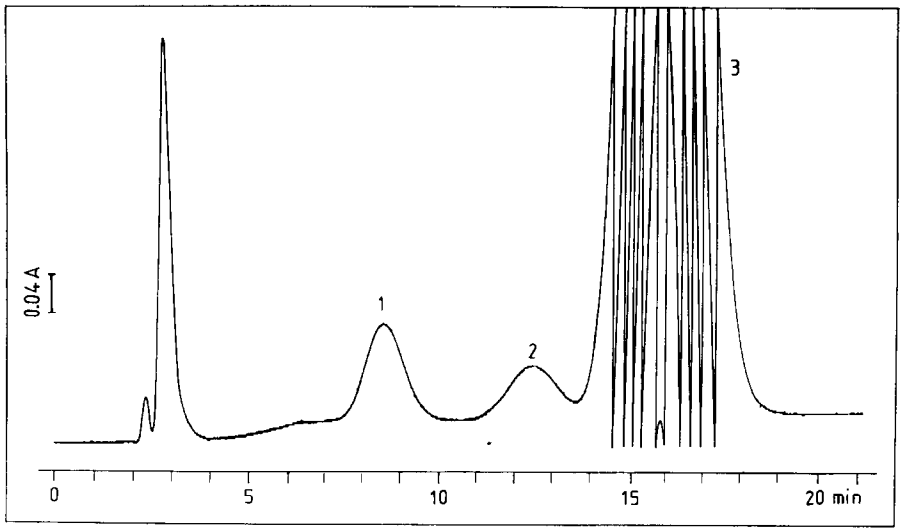

Fig. 13. Analysis of a commercial preparation of adenosine-5′-triphosphate by chromatography on DE-AE-cellulose. Column: 30 × 1 mm I.D. Linear gradient of potassium phosphate, pH 7.5 (0–0.2 *M*) in 7 *M* urea, 1100 μl. Flow-rate: 20 μl/min. Detection at 260 nm. 1 = AMP; 2 = ADP; 3 = ATP.

possible to run chromatography in highly aggressive media. An example of such a separation is the chromatography of aromatic amines on Dowex 50 cation exchanger in a linear gradient of hydrochloric acid (Fig. 12). Fig. 12 also illustrates the time-share principle of multi-wavelength detection. The good separation in this experiment is due to the high selectivity rather than to the high efficiency of the column.

Fig. 13 illustrates the application of the OB-4 to the analysis of a commercial preparation of adenosine-5′-triphosphate (ATP). The separation was made on an adsorbent that is not used in HPLC, but widely applied in biochemistry, DEAE-cellulose. Owing to the small length of the column (30 mm), the separation takes a relatively short time (20 min). Fig. 13 also illustrates the "scale folding" facility of the instrument. Depending on the position of the switch "fold scale" (position 9 in Fig. 1), the pen of the recorder having arrived at the highest position will either remain there while the absorbance is off-scale, or return to the lowest position and continue the record with one full scale subtracted. The first mode is more useful in multi-wavelength detection of off-scaling peaks. The second mode is used in single-wavelength detection for the analysis of small admixtures. In this particular instance (Fig. 13), the admixtures of AMP and ADP were 2.5 and 2.0%, respectively, as calculated easily from the peak heights and the number of off-scales of the main peak. The minimum admixture that can be detected in this way is about 0.05%.

One more example of the application of a column of high selectivity, but low efficiency, is the rapid separation of AMP, ADP and ATP on silica gel coated with polyethyleneimine (Fig. 14). This separation took 1.5 min, and the pressure was only 1.5 MPa.

Chromatography on highly efficient columns

This type of chromatography is performed using stainless-steel columns (Fig.

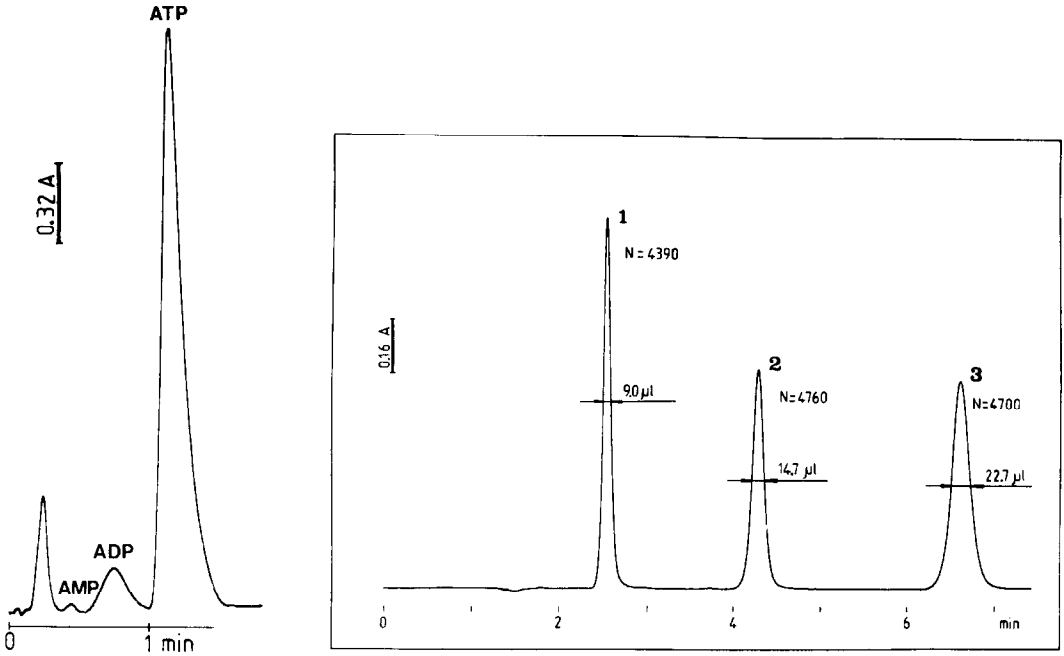

Fig. 14. Chromatography of adenosine-5′-mono-, -di- and -triphosphates on silica gel coated with polyethyleneimine (the adsorbent was kindly provided by Dr. V. P. Kumarev). Column: 50 × 1 mm I.D. Linear gradient of Tris–HCl, pH 8 (0.2–1.2 *M*), 1100 µl. Flow-rate: 600 µl/min. Detection at 260 nm.

Fig. 15. Test for the efficiency of a microcolumn. Chromatography of a model mixture of nitroanilines (1 = *ortho*-; 2 = *meta*-; 3 = *para*-). Column: 62 × 2 mm I.D., Silasorb 600, 5-µm particles. Sample: 1 µl, 1 µg of each of the compounds. Eluent: hexane–chloroform–2-propanol (70:22:8). Flow-rate: 100 µl/min. Detection at 290 nm.

4B). The experiments described below were all made on standard 62 mm × 2 mm I.D. columns packed with 5 µm adsorbent particles. The packing was made at 25 MPa using the upstream slurry procedure. To date, many highly efficient procedures have been elaborated for the application of the OB-4 in different fields. The examples presented below are only an illustration of the performance of the OB-4.

Fig. 15 shows the result of the separation of a model mixture of nitroanilines on a silica gel column. It can be seen that the total efficiency of the column is close to 5000 theoretical plates.

Fig. 16 shows a chromatogram of the same mixture detected at a short wavelength well below the "cut-off limit"; the absorbance of the solvent in this experiment in a 1 cm cell would be more than 10.

The silica gel column used in the above experiments has been employed to separate many important mixtures, mainly of organic synthesis intermediates.

Saturation of silica gel with copper–ammonia solution gives an efficient ligand-exchange column that can be used for the chromatography of amino acids, peptides and other compounds[16]. Fig. 17 shows an example of the application of this column to the separation of amino acids. It is interesting that the peaks of different amino

acids (they are eluted as copper coordination compounds) have different absorbance ratios. This property simplifies their identification.

Fig. 18 shows the result of the reversed-phase chromatography of some aromatic hydrocarbons. In this instance, detection at two wavelengths again facilitates identification.

One more example of reversed-phase chromatography is shown in Fig. 19, which illustrates the direct analysis of human blood serum for the content of uric acid. In this experiment, 5 μl of untreated serum were applied to the column. Owing to detection at two wavelengths, the identification again does not leave any doubts, although the retention time of uric acid in the real sample is different from that found in the standard.

Fig. 20 shows the result of the separation of a mixture of phenylthiohydantoin (PTH) derivatives of amino acids by reversed-phase chromatography. In both instances (A and B) the amounts of PTH-amino acids taken for analysis were similar (100–200 pmole). Fig. 20A shows a chromatogram obtained with the OB-4, and Fig. 20B a chromatogram obtained with the "normal-size" high-performance liquid chromatograph of a commercially available peptide sequenator. Comparison of these two chromatograms leads to the following conclusions: (i) the resolution obtained with

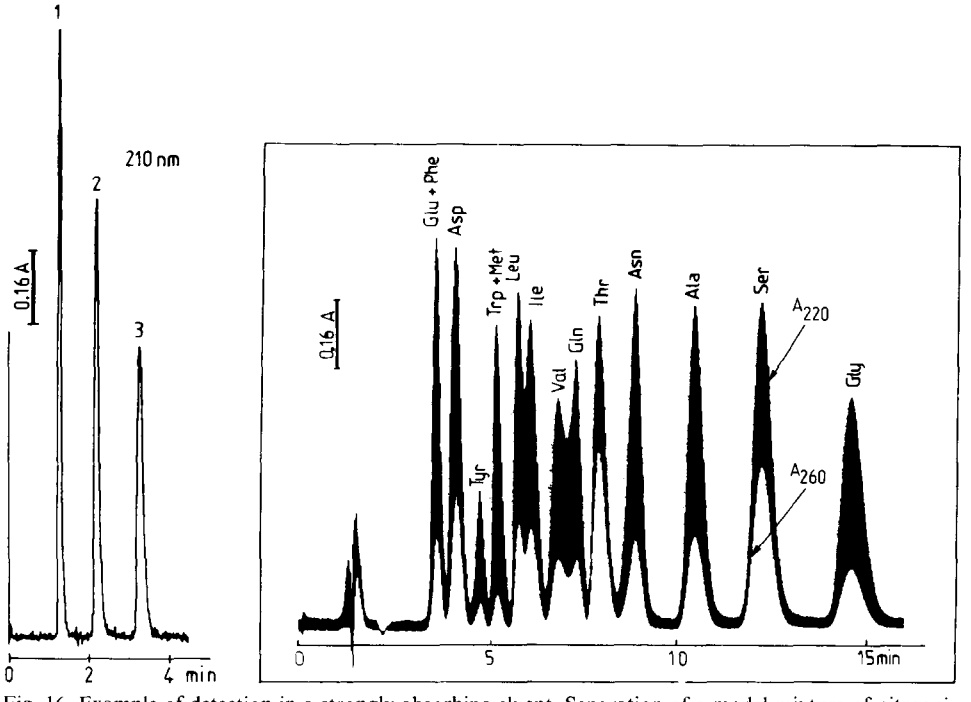

Fig. 16. Example of detection in a strongly absorbing eluent. Separation of a model mixture of nitroanilines (2 μl, 2 μg of each). Column as in Fig. 15, but less efficient. Flow-rate: 200 μl/min. Eluent: hexane–chloroform–2-propanol (70:22:8). Detection at 210 nm.

Fig. 17. Separation of amino acids on a silica gel column saturated with copper–ammonia complex[16]. Column: 62 × 2 mm Silasorb 600 treated as described in ref. 17. Eluent: water–acetonitrile (40:60)–0.35 *M* ammonia; flow-rate, 100 μl/min. Detection at 220 and 260 nm. Sample: Trp, Tyr, Asp, Glu and Phe *ca.* 2 nmoles each, other amino acids *ca.* 100 nmoles each, in 10 μl of water.

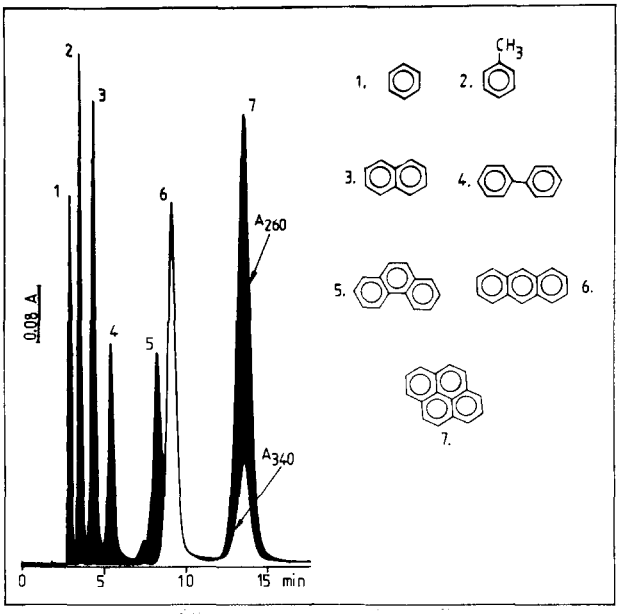

Fig. 18. Reversed-phase chromatography of aromatic hydrocarbons. Column: 62 × 2 mm I.D., Nucleosil C_{18}, 5-μm particles. Eluent: methanol–water (80:20). Flow-rate: 50 μl/min. Detection at 280 and 340 nm. Sample: 0.5–5 μg of hydrocarbons in 2 μl of methanol.

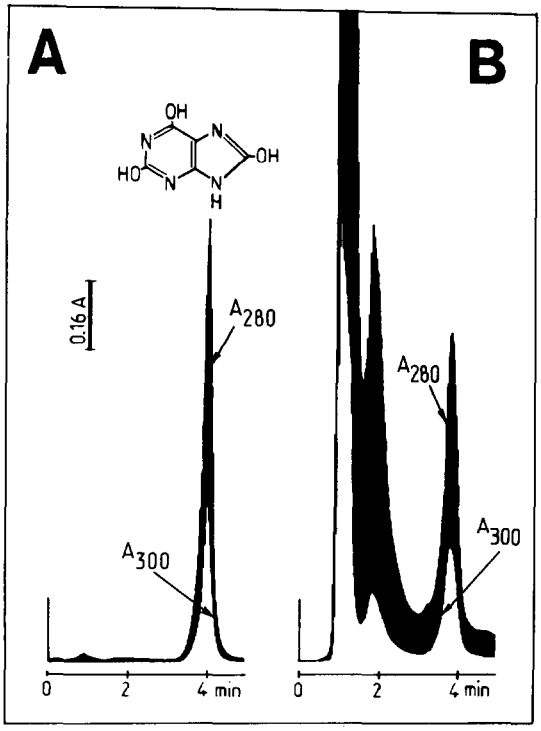

Fig. 19. Direct analysis of human serum for uric acid by reversed-phase chromatography. Column: 62 × 2 mm I.D. Nucleosil C_{18}. 5-μm particles. Eluent: 4% methanol in 0.01 *M* acetic acid. Flow-rate: 100 μl/min. Detection at 280 and 300 nm. A, Standard, 5 μg of uric acid in water; B, 5 μl of normal human blood serum.

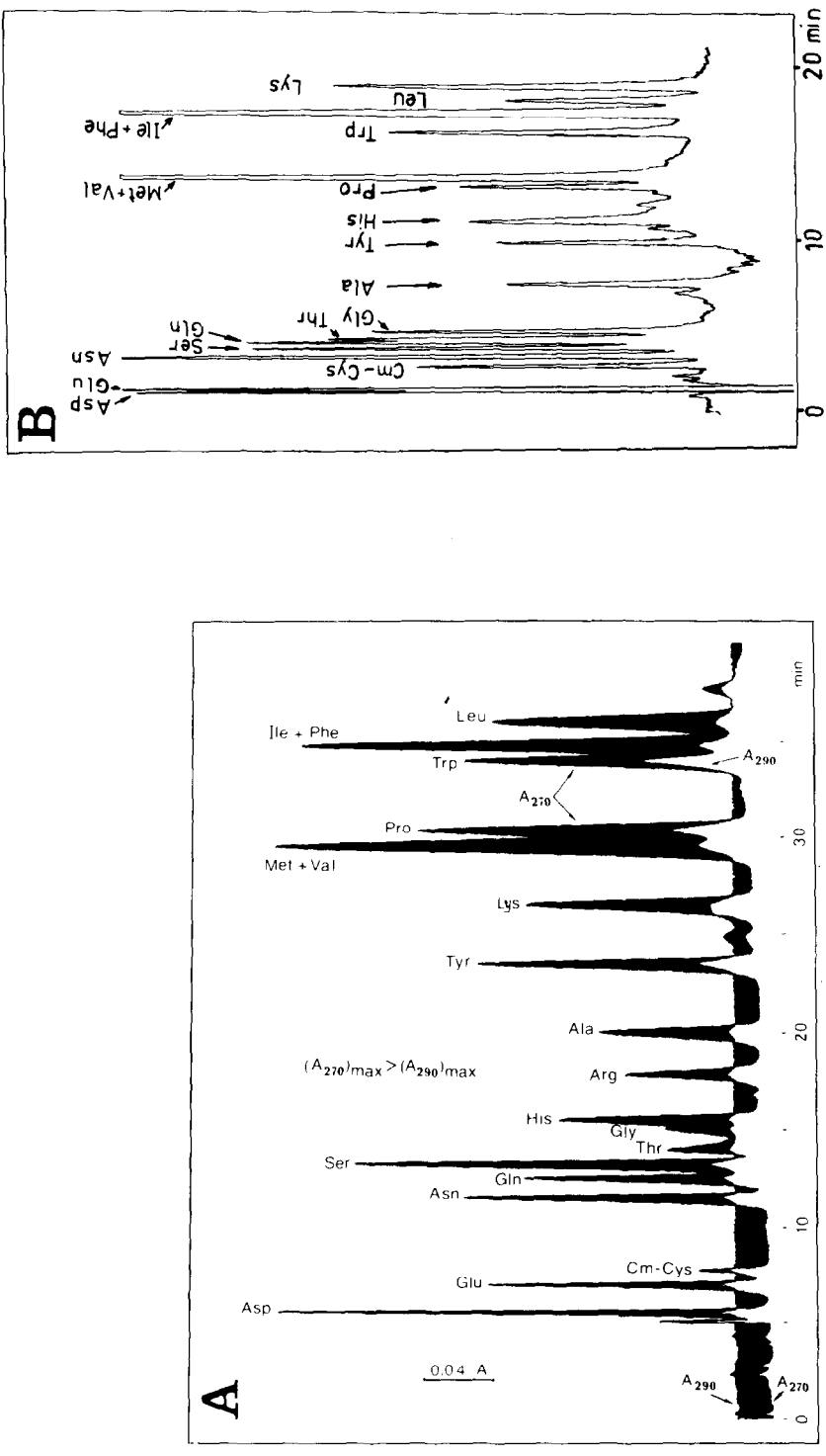

Fig. 20. Reversed-phase chromatography of phenylthiohydantoin derivatives of amino acids, *ca.* 200 pmole (40 ng) each. A. Chromatogram obtained with the OB-4. Column: 62 × 2 mm I.D., Nucleosil C_{18}, 5-μm particles. Eluent: linear gradient of acetonitrile (20–50%) in 0.01 *M* ammonium acetate (pH 5.8). Flow-rate: 50 μl/min. Detection at 270 and 290 nm. B. Chromatogram obtained with the MP-100 PTH-analyser of the Sequemat (Boston, MA, U.S.A.) peptide sequencer. Column: 150 × 4.6 mm I.D. Ultrasphere ODS C_{18}, 5-μm particles. Eluent: linear gradient, buffer A to buffer B. Flow-rate: 1.5 ml/min. Detection at 270 nm. Buffer A: 220 ml of methanol, 110 ml of acetonitrile, 50 ml 0.06 *M* sodium trifluoroacetate (pH 6.0), water to 1 l. Buffer B: 220 ml of methanol, 300 ml of acetonitrile, 140 ml 0.06 *M* sodium trifluoroacetate (pH 3.4), water to 1 l.

the 200-μl micro-column of the OB-4 is not very different from that obtained with the 2500-μl "normal-size" column; (ii) detection at two wavelengths with the OB-4 helps to identify some PTH-amino acids more reliably; (iii) the consumption of solvent with the OB-4 is about 15 times smaller; (iv) owing to the higher concentration of PTH-amino acids which comes to the detector in micro-column HPLC, the chemical noise (the instability of the baseline) is much smaller with the OB-4; obviously, the procedure illustrated in Fig. 20A is much further from its sensitivity limit than the procedure illustrated by Fig. 20B.

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