Journal of Chromatography, 450 (1988) 81–89
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1410

ECONOMIC LABORATORY PRACTICE IN PREPARATIVE COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

G. FRANKE
Abimed Analysen Technik GmbH, Postfach 2141, 4018 Langenfeld (F.R.G.)
and
F. VERILLON*
Gilson Medical Electronics S.A., B.P. 45, 72 Rue Gambetta, 95400 Villiers le Bel (France)

SUMMARY

For isolating from 1 mg up to 200 g of pure substance from unknown and complex mixtures, a set of materials and methods is presented and evaluated by several applications. The procedure combines automated sequential high-performance liquid chromatography with gradient elution on a 50 mm × 21.4 mm, 3-μm packed column. The method development time is very short and the chromatographic system has an high specific production rate. The preparative performance is discussed, and compared with other approaches.

INTRODUCTION

On the laboratory scale, flexible automation is required in column liquid chromatography to accommodate frequent application changes and variable quantity ranges. Technical optimization is less critical than for industrial-size separations, but economic aspects are seldom negligible. For isolating several grams of pure substance from unknown and complex mixtures, the time is considerably reduced by using a 3-μm packed short column of about 20 mm I.D., supplemented by an automatic system monitoring the repeatability of unattended operations. The same instruments may also be used for analytical purposes and in a research environment. A method is presented with practical details and several applications are described. The performance is discussed, and compared with other approaches.

Preparative column liquid chromatography (PCLC) may be classified according to the order of magnitude into four groups, as shown in Table I. Micropreparative chromatography isolates subnanomole quantities of proteins and polypeptides for microsequence analysis[1,2], while macropreparative fractionation may reach kilogram levels. This upper limit corresponds to a maximum column diameter of about 200–300 mm, and a maximum flow-rate of about 5 l/min. At the other end of the range, the minimum preparative sizes are continuously being lowered, just as upper limits are rising in industrial applications. The experiments reported here exclude micropreparative work and do not exceed 200 g of collected mass, column diameter of 41.4 mm and flow-rates of 50 ml/min.

0021-9673/88/$03.50

TABLE I

SIZE CLASSIFICATION OF LABORATORY-SCALE PCLC

Collected mass (g)	*Designation*	*Column diameter (mm)*	*Purpose*
$<10^{-6}$	Micropreparative	<2	Bioanalysis
10^{-6}–10^{-3}	Semipreparative	2–10	Chemical analysis
10^{-3}–1	Preparative	10–50	Identification and reactions
1–10^{3}	Macropreparative	20–300	Manufacture of standards

EXPERIMENTAL

Materials

The chromatograph used was a binary-gradient, automatic, preparative system (Gilson Medical Electronics, Villiers le Bel, France). It consisted of two elution pumps (303 with a 25- or 50-ml/min pump head), injection pump (302 with a 10-ml/min pump head) and a manual injection valve with 0.5-ml loop (7125; Rheodyne, Cotati, CA, U.S.A.), a manometric module (803 C), dynamic mixer (811 A), UV-absorbance detector with 0.2-mm lightpath cell (116), a fraction collector with a three-port valve (202 C), system controller (IBM AT with hard disk, EGA graphic card, mouse, MS DOS 3.1 and Windows software), contact module (506), system software (714) and printer (Hewlett-Packard Thinkjet). This equipment is characterized by a modular hydraulic structure and programmable operating functions.

All columns were Dynamax (Rainin Instrument, Woburn, MA, U.S.A.), from 4.6 to 41.4 mm I.D., cartridge-type replaceable columns and guard modules, with permanent, axial-compression end-fittings and replaceable inlet filter. The method described is based on the direct use, as a separation column, of a 50 mm × 21.4 mm guard module, packed with 3-μm stationary phase, equipped with a 0.5-μm inlet filter and installed inside a permanent holder. Solvents, from various sources, were either of high-performance liquid chromatography (HPLC) grade or distilled, according to the application, and always filtered through 0.5-μm filters.

Method

The method was developed to isolate several grams of pure substances from unknown and complex mixtures. In this type of separation problem, the components which have already been identified are rare. Generally, only some indications of the chemical structure and functional groups are available. From this limited information, the chromatographer has to choose the separation phase system as well as the detection conditions. The procedure is based on the direct use of the 50 mm × 21.4 mm, 3-μm column, systematically under gradient elution. Such a column contains about 9 g of silica-based stationary phase and the total volume of the mobile phase inside is about 11 ml. Most mixtures were resolved on a reversed-phase octadecylsilane (ODS) column with water–methanol at ambient temperature. Under these conditions, the maximum pressure seldom exceeds 8 MPa (80 bar, 1100 p.s.i.) for a typical flow-rate of 15 ml/min. Two detection channels were generally used, either at two

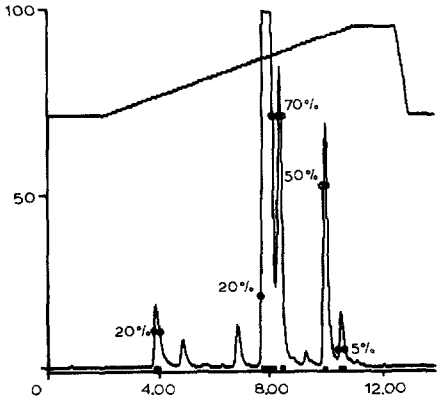

Fig. 1. Separation of phenolic esters. For details, see Table II, No. 3; detection, 300 nm, 10 a.u.f.s. Time scale in min. Signal and gradient profile in %.

different wavelengths (from 190 to 380 nm) or at two different sensitivities (from 0.025 to 50 a.u.f.s., related to a 10-mm lightpath). The unknown sample (solid or concentrated liquid mixture) was dissolved in a minimum volume of mobile phase having an elution strength equal to or below that of the initial gradient composition, in order to generate favourable effects of enrichment or peak compression. It was then filtered through a 0.5-μm filter. This sample solution had a typical concentration of 10 mg/ml. No other sample pretreatment was performed in the cases described.

The procedure included three basic steps. (1) Determination of the mobile phase flow-rate and gradient profile by manual sample injections of 0.5 ml. (2) Determination of the injection volume by automatic injections of increasing volumes, approaching volume or mass overload, but generally not higher than the column linear capacity threshold. This volume reached 5 ml in favourable cases. (3) Unattended, multicycle, preparative separation, with fraction collection combining time and signal criteria. When the objective was to purify one or a few major components, as opposed to isolating trace components or impurities, the last step was preceded by a tentative optimization of fractionation cut points, based on a compromise between purity and recovery. Variable fractionation points were selected from the chromatogram displayed on the screen, as illustrated by Fig. 1. The instrument design also allowed us to choose the most appropriate collection vessels, generally 250-ml round-bottom flasks. Purity tests of collected fractions were directly performed by manual injections of 0.5 ml of the collected solutions, on the same column and under a 10- to 100-fold higher detection sensitivity.

RESULTS AND DISCUSSION

These methods have been extensively applied to isolate from 1 mg to 200 g of pure substances from natural products, plant protective agents, pharmaceutical compounds, synthetic oligopeptides and metal complexes. Some of these applications are presented in Table II (separations 1–9 on column 1) where they can be compared with others, performed on columns of closely related, as well as very different, dimensions.

TABLE II

PCLC SEPARATIONS ON ODS COLUMNS BY GRADIENT ELUTION WITH WATER AND METHANOL

Exceptions: 9 and 16, water and acetonitrile on ODS (with 0.1% tetrahydrofuran for 9); 7, *n*-hexane and dichloromethane on silica; 13, *n*-hexane and methyl *tert.*-butyl ether on silica; 14 and 15, methyl *tert.*-butyl ether and methanol on silica; 17 and 18, potassium phosphate (pH 6.0) from 5 to 300 m*M* on Dynamax AX (anion exchanger).

No.	*Application*	*Flow-rate (ml/min)*	*Injected volume (ml)*	*Injected mass (mg)*	*Cycle time (min)*	*Throughput (mg/min)*	*Specific throughput (mg/g · l · h)*	*No. of compounds collected/detected*
Column 1: 50 mm × 21.4 mm, 3 μm, 100 Å (*ca.* 3000 theoretical plates)								
1	A: Lavender oil	15	2	20	15	1.3	40	5/12
2	B: Peppermint oil	15	5	125	10	12.5	560	3/16
3	C: Phenolic esters	10	1	10	13	0.8	30	5/14
4	D: Triptycenes	15	2.5	25	12	2.1	62	5/18
5	E: Analgesic	10	2	120	20	6.0	200	3/24
6	F: Iron complexes	10	0.5	5	10	0.5	33	4/16
7	F: Iron complexes	15	1	12	15	0.8	24	5/21
8	G: Cerebrosides	20	1	25	10	2.5	83	10/19
9	H: Oligopeptides	20	1	25	14	1.8	43	5/41
Column 2: 100 mm × 21.4 mm, 3 μm, 100 Å (*ca.* 8300 theoretical plates)								
10	I: Camomile oil	15	5	50	30	1.7	12	3/40
11	J: Perfume	15	5	50	40	1.2	7	6/44
12	K: Eucalyptus oil	15	0.5	25	30	0.8	6	8/12
Column 3: 50 mm × 21.4 mm, 8 μm, 60 Å (*ca.* 1600 theoretical plates)								
13	L: Insecticide	24	0.2	250	14	17.9	355	1/8
Column 4: (50 + 250) mm × 21.4 mm, 8 μm, 60 Å (*ca.* 9000 theoretical plates)								
14	M: Pesticide	24	0.4	120	20	6.0	14	1/7
15	N: Insecticide	24	1.5	110	18	6.1	16	2/2
16	O: Fungicide	24	1.5	75	72	1.0	1	9/17
Column 5: 250 mm × 41.4 mm, 12 μm, 300 Å (*ca.* 5000 theoretical plates)								
17	P: Lactoglobulins	49	30	1300	45	28.9	5	4/8
18	Q: α-Lactalbumin	49	40	2000	65	30.8	3	1/32

Preparative performance

The results of preparative separations are currently expressed in terms of the throughput (TP) and production rate (PR). This second parameter is of prime importance for the industrial scale, where one is dealing with known mixtures. The throughput, *i.e.*, injected sample mass divided by separation cycle time, varies from 0.5 to 12.5 mg/min for separations 1–9. It is mainly a function of the sample solubility (10–60 mg/ml), number of compounds detected (12–41), and number of compounds collected (3–10). However, neither TP nor PR evaluates the chromatographic performance. Table II indicates, as a criterion of preparative performance, the specific throughput (STP), defined as the throughput divided by the mass of stationary phase and by the volume of mobile phase

$$\mathrm{STP} = 4m_s/\pi d_c^2 L\rho F t^2$$

where m_s is the sample mass injected, d_c and L the column inner diameter and length,

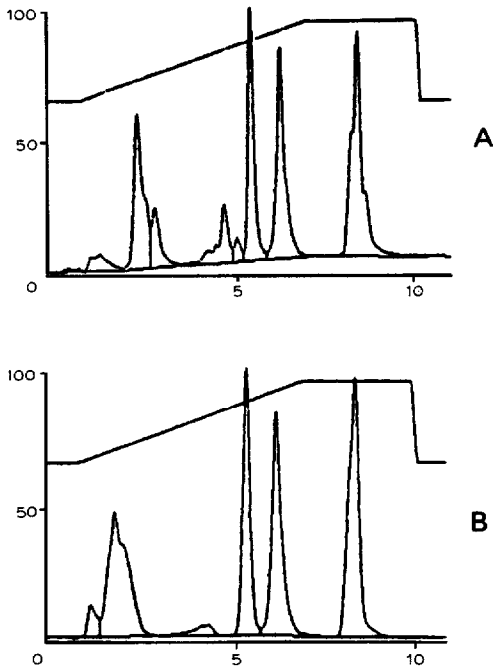

Fig. 2. Loadability test of peppermint oil. For details, see Table II, no. 2. (A) Concentration of injected solution is 1% of oil in 60% aqueous methanol; 0.5 ml, containing 5 mg, are injected and detected at 230 nm, 2 a.u.f.s. (B) Concentration of injected solution is 2.5% of oil in 60% aqueous methanol (emulsion); 5 ml, containing 125 mg, are injected and detected at 230 nm, 30 a.u.f.s. Time scale in min. Signal and gradient profile in %.

ρ the stationary-phase packing density (0.5 g/ml, assumed to be constant in Table II), F the mobile phase flow-rate, and t the separation cycle time. STP is thus the sample load of the phase system per time unit. It is much higher for experiments 1–9 than for the other experiments reported in Table II, except for 13, which will be discussed further. This index has been already used[3] to demonstrate the benefits of the recycling technique[4] compared with single-pass separations. At that time, the best value was only 5 mg/g · l · h, obtained for the separation of two isomers, from 2.3 g, in 1 h, on a 500 mm × 32 mm, 11-μm column, with mass overload and five-cycle recycling. Today, in the case of experiment 2, STP is more than 100 times higher. Two extreme chromatograms, performed as loadability tests for this separation, are shown in Fig. 2. Similarly, the production rate of a compound, *i.e.*, collected fraction mass, m_f, divided by separation cycle time, can be related to the amounts of chromatographic phases and is called the specific production rate (SPR) for this compound. The corresponding expression is

$$\text{SPR} = 4m_f/\pi d_c^2 L\rho F t^2 n$$

where n is the number of injections corresponding to m_f. SPR is 10 mg/g · l · h for experiment 15 which produced 50 g of pure insecticide in 700 cycles with a yield of 70%. It is more than 200 mg/g · l · h for experiment 13 which produced 190 g of another

insecticide, purified from 76 to 99%, in 1200 cycles, *i.e.*, 12 days. This second example is considered as being the ultimate in productivity. The mobile phase composition and flow-rate were optimized, and the liquid sample was injected without being previously dissolved. At the other extreme, experiment 9 produced about 1 mg of each collected oligopeptide in 25 min.

Benefits of a short column, packed with fine particles

Already popular as "fast" HPLC on 4- to 5-mm diameter columns, the advantages of 3-μm packed, short columns are especially appropriate for PCLC, featuring short separations times (10–20 min), low solvent consumption (100–200 ml per cycle on a 21.4-mm diameter column) and high solute concentration. Moreover, the substance concentration is further increased by gradient elution, and gradient elution benefits from rapid column reconditioning (2–3 min). A solvent change requires about 5 min. With silica, three preliminary cycles are enough to stabilize the system. In addition, the fact that up to 200 g can be purified with only 9 g of stationary phase may justify the use of very selective and expensive phases, especially in affinity chromatography, for producing antibodies and enzymes.

Other benefits concern method development and method transfer. With the above-described procedure, the time needed for method development was 3–5 h, with typically 15–20 injections, including 10 manual ones. If greater efficiency is needed, transfer from column 1 to column 2 or 4 is also very rapid, usually limited to a proportional adaptation of the time points of the gradient profile to the length and flow-rate of the new column. If an higher throughput is desired, transfer from column 1 to column 5 should solve the problem. It would require only a change of pump head on the two elution pumps. Experiment 13 shows that a very satisfactory result is achieved on column 3, having 8-μm instead of 3-μm particles.

Fig. 3 presents Van Deemter curves obtained with preparative injections into

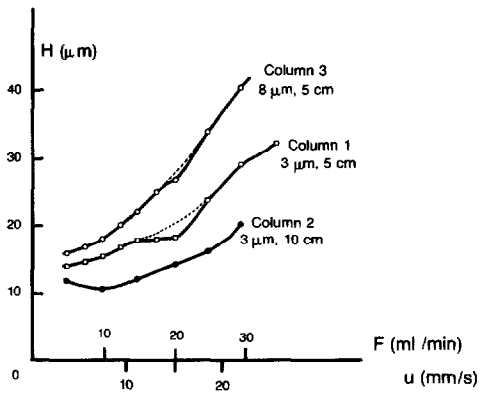

Fig. 3. Plate height (H) *versus* linear velocity (u). Columns: 1 = Rainin, 50 mm × 21.4 mm, 3-μm Microsorb C_{18}, spherical, 100 Å; 2 = Rainin, 100 mm × 21.4 mm, 3-μm Microsorb C_{18}, spherical, 100 Å; 3 = Rainin, 50 mm × 21.4 mm, 8-μm Dynamax C_{18}, irregular, 60 Å. Mobile phase; 80% aqueous methanol, isocratic; injected volume, 0.5 ml; sample, test mixture, containing 0.01 g/l uracil, 0.02 g/l acetophenone, 0.40 g/l anisole and 1.13 g/l toluol, dissolved in mobile phase; reference peak, toluol (k' = 1.6 on columns 1 and 2, 2.0 on column 3); detection, 254 nm, 0.2 a.u.f.s. F = Flow-rate.

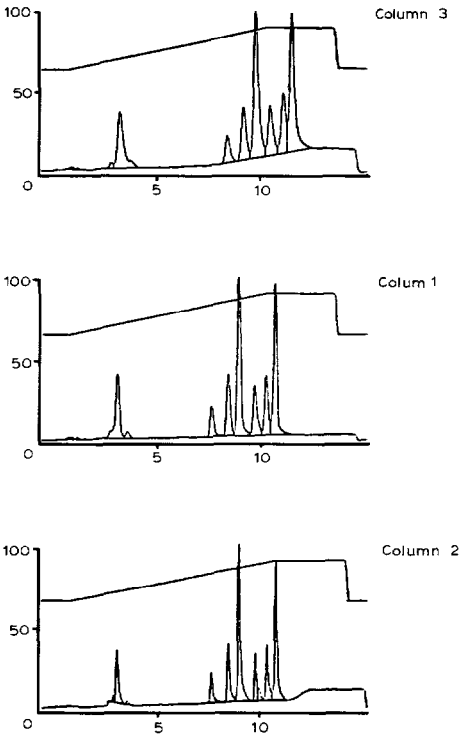

Fig. 4. Column comparison. Columns 1–3 as in Fig. 3; mobile phase, gradient from 65 to 90% of methanol in water; flow-rate, 1.5 ml/min; injected sample, 0.5 ml, containing 25 mg of eucalyptus oil; detection, 230 nm, 3 a.u.f.s. Time scale in min. Signal and gradient profile in %.

columns 1, 2 and 3 (Table II). The distortions of curves 1 and 3 may be attributed to temperature effects, as mentioned by Verzele and Dewaele[5], and also to the column technology, since column 2, in contrast with the other two, is not of the cartridge type with an holder, *i.e.*, it has a single instead of a double wall. These three columns have been compared in the separation of eucalyptus oil (Fig. 4). This showed that column 1 is preferred. Other chromatograms are presented in Figs. 5 and 6.

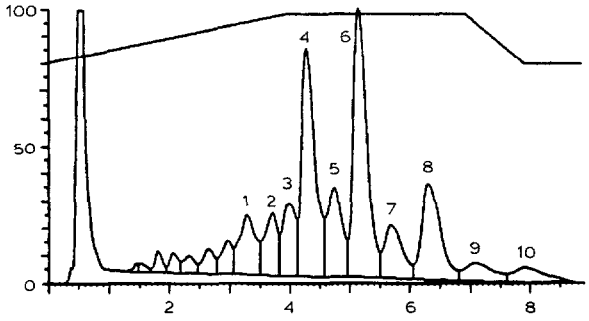

Fig. 5. Separation of cerebrosides. For details, see Table II, No. 8; detection, 230 nm, 1 a.u.f.s.; a silica thin-layer chromatogram of this sample revealed only three spots. Time scale in min. Signal and gradient profile in %.

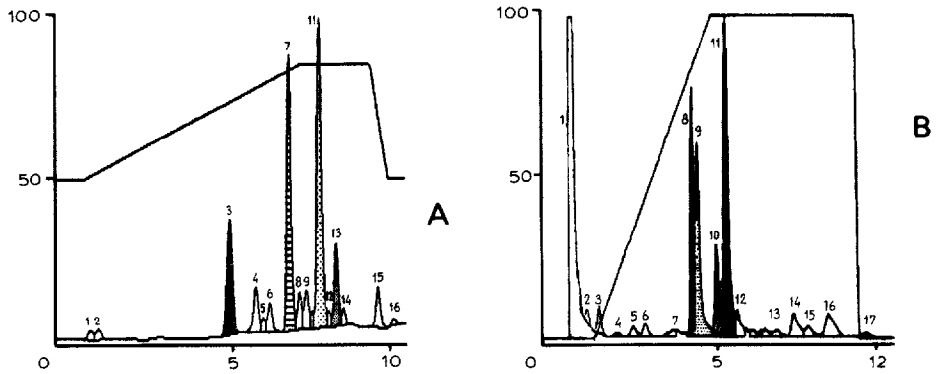

Fig. 6. Separation of iron complexes. (A) For details, see Table II, No. 6; detection, 254 nm, 0.1 a.u.f.s. (B) For details, see Table II, No. 7; detection, 254 nm, 0.5 a.u.f.s. Time scale in min. Signal and gradient profile in %.

Automation

After method development (1/2 day) the chromatograph operates unattended, generally during the other half day for 1 g, and up to *ca.* 1 week for up to 100–200 g. In order to operate for 168 h a week instead of just 40 h, the instrument requires several fail-safe features and an high level of repeatability. The requirements for the fraction collector have already been examined in detail[6]. The model chosen is capable of interrupting the system in the case of retention shifts and baseline drifts, in accordance with continuously adjustable parameters. It also has provision for flushing the module with an inert gas. The system software can activate a predefined emergency programme from a contact closure in case of failure or a pressure outside of predefined limits. The column is unbreakable. There is no need to degas the solvents, the pump heads are not sensitive to air-bubbles (the high-pressure mixing eliminates them), and

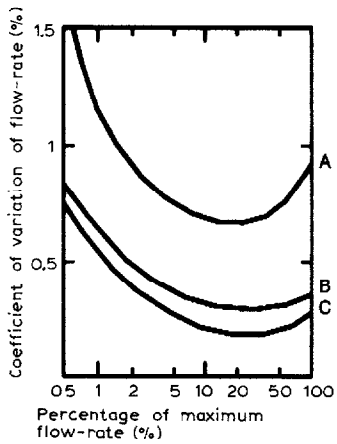

Fig. 7. Precision of Gilson piston pumps. (A) Chloroform (viscosity, 0.57 cP at 20°C); (B) water (1.0 cP at 20°C); (C) 60% aqueous methanol (1.8 cP at 20°C). Measurements were made by volumetry, gravimetry and chromatography from retention times. Each curve was drawn from 21 points and each point represented from 5 to 50 measurements. The deviation of experimental points from each curve was less than 25%. These curves are valid for all models of interchangeable pump heads, at ambient temperatures and under any positive pressure below the specified limit.

the fraction collector is able to neglect possible air spikes thanks to an adjustable time-constant filter.

Lastly, the repeatability of each cycle mainly depends on the pumping system. Fig. 7 shows precision curves, obtained with the pumps used for the experiments described. Repeatability of the flow-rate as well as of the injected volume depends both on the nature of the solvents and on the flow-rate selected. The column is very stable over 1000 cycles. Loss of efficiency was seldom observed and generally well compensated for by hand-tightening of the axial compression fittings.

CONCLUSION

As a solution for isolating from 1 mg to 200 g of pure substances from unknown and complex mixtures, the procedure presented implements automated sequential HPLC with gradient elution from a 50 mm × 21.4 mm, 3-μm column. This small column has an outstandingly high production rate. Having completed method development, the chromatographer can leave the instrument to operate unattended for up to 10 days. The same instrument is used to check the purity of collected fractions and is compatible with current-scale analytical HPLC. The column cost, solvent consumption, labour and investment are considerably lower with such a procedure than with manual injections into long columns, packed with coarser particles. While analytical separation is a measuring process, preparative separation is in essence a measured operation, where economy factors are meaningful at any scale.

REFERENCES

1 E. C. Nice and R. J. Simpson, *Chromatographia*, 24 (1987) 449.
2 H. Kotaniguchi and M. Kawakatsu, *Int. Lab. (Asian Ed.)*, 3 (1988) 20.
3 F. Verillon, *Ph.D. Thesis*, Université P. et M. Curie, Paris, 1977.
4 M. Martin, F. Verillon, C. Eon and G. Guiochon, *J. Chromatogr.*, 125 (1976) 17.
5 M. Verzele and C. Dewaele, in *Preparative High Performance Liquid Chromatography: A Practical Guideline*, TEC, Gent, 1986, p. 50.
6 J. Krohn and F. Verillon, *Int. Lab.*, 16 (1986) 46.