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A LOW-COST GRADIENT SYSTEM FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

QUANTITATION OF COMPLEX PHARMACEUTICAL RAW MATERIALS

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SUMMARY

A device is described that makes use of an eight-port motor valve to generate step gradients on the low-pressure side of a piston pump with a low dead volume. Such a gradient device with an automatic control unit, which also permits repetition of previous steps, can be built for about half the cost of a gradient system with two pumps.

Applications of this gradient unit to the separation of complex mixtures of glycosides and alkaloids are discussed and compared with separations with commercial gradient systems using two high-pressure pumps. The gradients that are used on reversed-phase material with solvent mixtures of water and completely miscible organic solvents are suitable for quantitative routine control of pharmaceutical products. The reproducibility of retention data is excellent over several months and, with the use of loop injectors, major components can be determined quantitatively with a reproducibility of better than 2% (relative standard deviation).

The step gradient selector valve can also be used as an introduction system for very large sample volumes. Up to 1 l can be injected and samples with concentrations of less than 1 ppb can be determined with good reproducibilities.

INTRODUCTION

Gradient elution techniques in liquid chromatography are of prime importance for the separation of complex mixtures with widely varying polarities. A review on gradient elution systems for high-performance liquid chromatography (HPLC) was published by Veening[1]. Most commercial gradient systems generate the gradient on the high-pressure side, which requires two high-quality pumps. Less costly devices are possible with the generation of the gradient on the low-pressure (atmospheric pressure) side.

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Low-pressure gradient devices have been discussed[2] but few commercial systems are really applicable to HPLC. Probably the most useful approach is that proposed by Scott and Kucera[3–5] and can be termed incremental gradient elution. Up to 20 solvent reservoirs have been connected to a 20-port PTFE rotary valve and mixing chamber. With such a device, separations ranging from squalene to sugars were possible in one chromatogram[3,4].

The system discussed in this work operates on a similar principle; it is demonstrated, however, that step gradients can be carried out in a much simpler fashion, at reasonable cost and with sufficient reliability for routine quantitative work.

INSTRUMENTATION

A scheme of the apparatus is shown in Fig. 1. It consists of a multi-port valve, preferably a motor valve for automation, a pump and a gradient control unit or programmer. No additional mixing chamber is required. The special requirements for these components are as follows. The valve should be suitable for automatic operation. It should have a small dead volume, a short switching time, be gas-proof when operating below atmospheric pressure and have as many ports as possible. For the pumps, it is important that the dead volume of the total system, including the pressure damping device, is small. The dependence of the flow-rate on the viscosity of the mobile phase should be negligible.

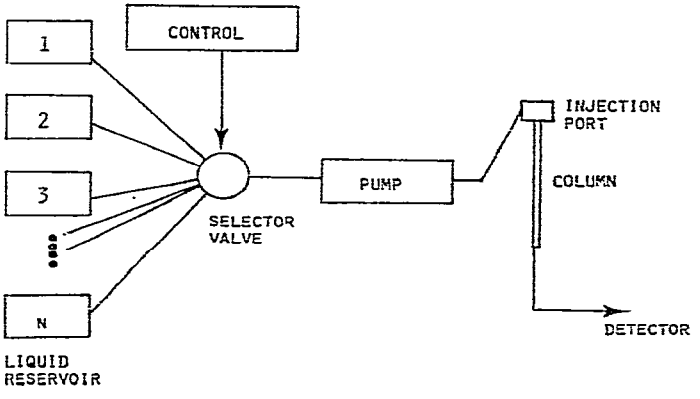

Fig. 1. Schematic diagram of step gradient system.

In this work, a Waters M6000 (Waters Assoc., Milford, Mass., U.S.A.) or an Altex Model 100 piston pump (Altex, Berkeley, Calif., U.S.A.) was used in combination with a Labotron 8-port motor valve cat. No. 002581 (Kontron, Zürich, Switzerland). An Altex Model 153 UV detector and a W + W Model 600 recorder (Kontron) were used in the chromatograph.

For automatic control of the gradient via the motor valve, mechanical or electronic timers can be used. These timers should possess the functions shown in Fig. 2. The first timer is started manually or by an automatic injection device. The

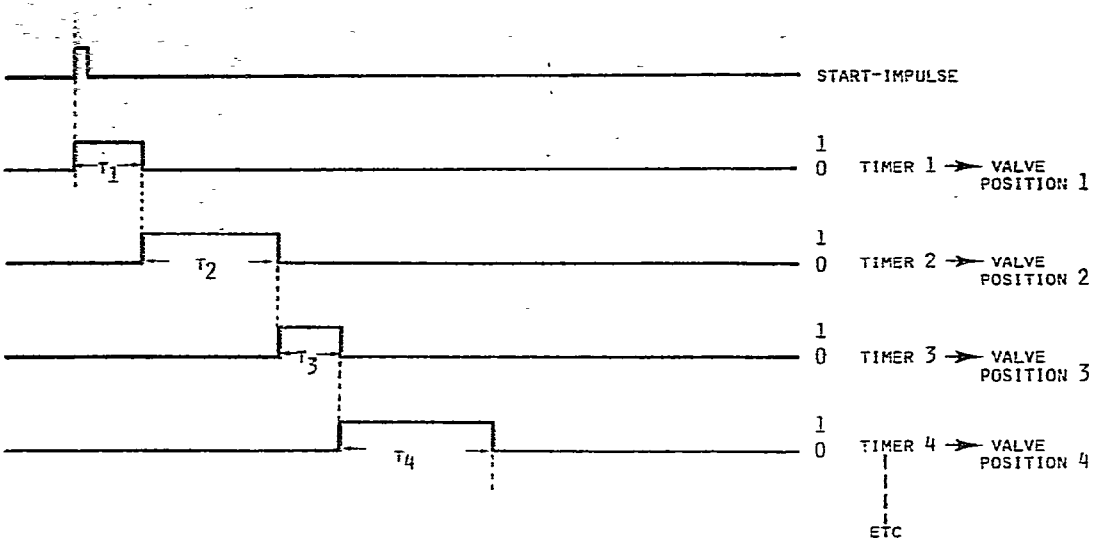

Fig. 2. Functional diagram of the step gradient programmer (control unit for Labotron valve).

starting impulse for the second time relay has to be given by the first and for the subsequent relays always by the preceding one. Type R.S. 21-XP 9 time relays (0.05 to 300 min) (Comatelectric, Worb, Switzerland) were used.

EXPERIMENTAL

The studies on gradient performance were carried out with solutions of caffeine as the UV chromophore in methanol, *n*-propanol and acetonitrile (analytical grade; Merck, Darmstadt, G.F.R.). Gradient profiles were measured without the use of a chromatographic column.

For practical applications, a plant extract of senna glycosides was separated on the reversed-phase material Nucleosil C_{18}, particle size 5 μm (Machery, Nagel & Co., Düren, G.F.R.) with Uvasol-grade acetonitrile (Merck) and a 0.01 M solution of sodium hydrogen carbonate. Direct injection of the extract was effected with a Valco loop, 7000 p.s.i., 30 μl.

A mixture of ergotamine (ergot alkaloid) and accompanying substances, ranging from lysergic acid to ergotaminine, was separated with an acetonitrile–0.01 *M* ammonium carbonate gradient on the same material. Details are given with the chromatograms. The quantitative evaluation of the chromatograms was carried out with an Infotronics Model CRS-204 integrator (Infotronics, Shannon Airport, Ireland) or the Hewlett-Packard Model 3352B data system.

RESULTS AND DISCUSSION

Gradient characteristics

The characteristic specifications were tested by using solutions of different concentrations of a UV-active substance. A detector signal proportional to the gradient profile can be obtained (Fig. 3).

The delay volume or time lag, which is the time from change of the valve posi-

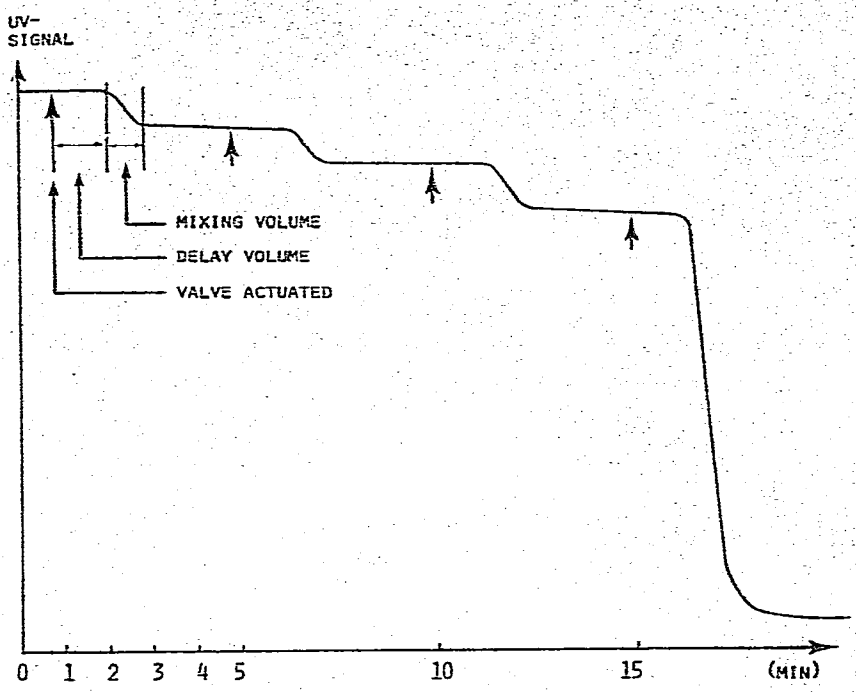

Fig. 3. Gradient profile of a step gradient system. Solutions with different concentrations of caffeine in methanol are mixed with the step gradient system without using a column.

tion to the first change in solvent composition at the detector, as well as the mixing volume can be seen in Fig. 3. The height of the step depends on the concentration difference of the various solutions. The mixing volume and the shape of the gradient profile are characteristic of this step gradient. A smooth linear gradient shape is observed for every solvent step. This shape differs favourably from the exponential dilution pattern obtained using a mixing chamber.

Investigations have shown that the delay volume is independent of the flow-rate and viscosity of the solvent. Tests were carried out with *n*-propanol with a viscosity of $\eta = 2.3$ cP and acetonitrile with $\eta = 0.37$ cP. The mixing volume, on the other hand, tends to decrease with increasing viscosity and to become more dependent on the flow-rate. The mixing volume for *n*-propanol, for example, which has a higher viscosity than acetonitrile, is about 40% less at a flow rate of 1 ml/min, 30% less at 2.5 ml/min and 10% less at 5 ml/min. No difficulties were encountered up to a flow-rate of about 4 ml/min. At higher flow-rates, the switching time of the motor valve becomes a problem. During the switching interval, the delivery of solvent to the pump is interrupted and a pressure reduction combined with the formation of air bubbles can affect the proper functioning of the pump.

Investigation of senna glycosides

The separation of a mixture of senna glycosides in a plant extract was carried out with the step gradient system described above, using five steps as indicated in Table I.

The separation achieved with this step gradient is shown in Fig. 4. In both

TABLE I

STEP GRADIENT PROGRAMME FOR THE SEPARATION OF SENNA GLYCOSIDES

Step	*Acetonitrile in 0.01 N $NaHCO_3$ (%)*	*Time programme*
1	1.50	Before injection
2	4.25	7 min
3	7.50	3 min
4	12.50	6 min
5	50	2 min

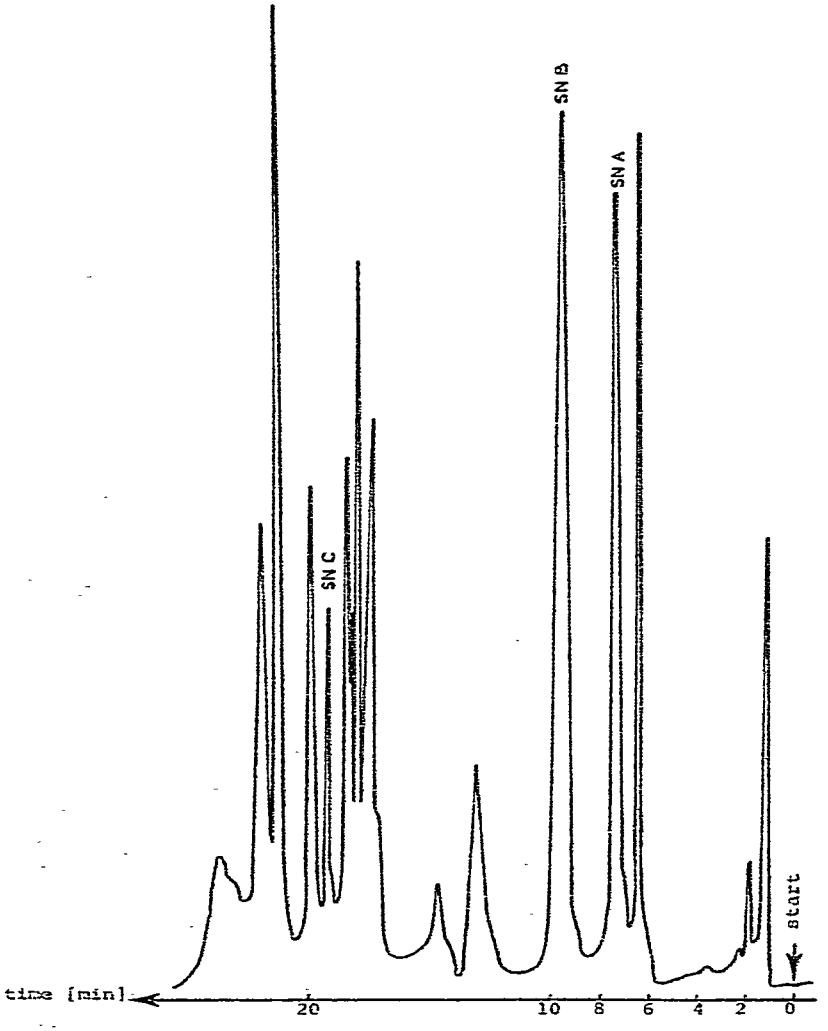

Fig. 4. Separation of senna glycosides in a plant extract with the step gradient system. Mobile phase, see Table I; other conditions as in Fig. 5.

instances a separation that permits the quantification of sennosides A, B (major active components) and C can be achieved in 12 min. The last gradient step has only a column cleaning function prior to starting the next chromatogram. The retention times are very reproducible over several months. The reproducibility for the quantitative determination of the content is good.

The relative standard deviations for a synthetic mixture of sennosides A, B and C were found to be as follows: sennoside A, $s_6 = 0.9\%$; sennoside B, $s_6 = 1.3\%$; sennoside C, $s_6 = 1.4\%$. With the actual plant extract they were higher owing to baseline variations that were not always ideally corrected by the electronic integrator: sennoside A, $s_3 = 1.6\%$; sennoside B, $s_3 = 1.3\%$; sennoside C, $s_3 = 2.4\%$.

The linear regression of the calibration graphs over a concentration range of two decades gave regression coefficients of 0.99970 for sennoside A and 0.99973 for sennoside B.

Investigation of ergot alkaloids

The separation of ergotamine and some of its accompanying substances on reversed-phase C_{18} material has been described by Bethke *et al.*[6]. The Waters Assoc. gradient system was used. The technique permits the complete separation and quantification of the active substance ergotamine and the compounds mentioned in Table II. It has been applied to the control of content and purity in ampoule solutions. An analogous separation was also achieved with the step gradient system discussed here (Fig. 5). A six-step programme was used and the separation was complete after 12 min. The reproducibility for the quantitative determination of 18 μg of ergotamine was 1.5% (relative standard deviation).

Time optimization can eventually yield a separation in 4.5 min (Fig. 6) without a critical loss in resolution. Only the separation of aci-ergotaminine and ergotamine becomes difficult but quantification is still possible.

The advantage of the reversed-phase system is a rapid reconditioning of the column to the initial conditions, which permits an immediate start of a new gradient cycle and analysis.

TABLE II

STANDARD DEVIATIONS OF CONTENT AND RETENTION TIME

Compare Fig. 6.

Compound	*Reproducibility*		
	Content	*Retention time*	
	s_6^{rel} (%)	s_6 (sec)	s_6^{rel} (%)
Main component			
Ergotamine	1.3	1.0	0.5
By-products and degradation products			
Lysergic acid	2.2	2.9	5.7
Isolysergic acid	3.6	3.2	3.0
Lysergic acid amide	1.8	2.7	1.8
Isolysergic acid amide	2.5	2.1	1.3
aci-Ergotamine	2.2	2.0	1.1
aci-Ergotaminine	2.3	1.4	0.7
Ergotaminine	5.4	0.8	0.3

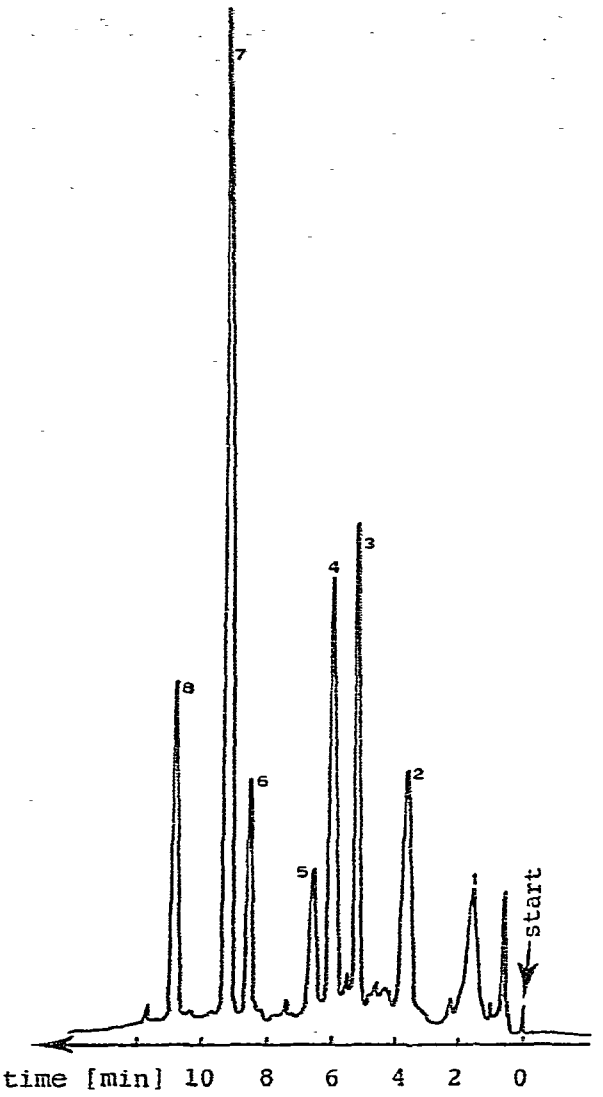

Fig. 5. Separation of ergot alkaloids. Mobile phase programme [acetonitrile–0.01 *M* $(NH_4)_2CO_3$ in water]: step 1, 8%, before injection; step 2, 15%, 1 min; step 3, 30%, 1 min; step 4, 40%, 3 min; step 5, 50%, 2 min; step 6, 60%, 4 min. Flow-rate, 1.5 ml/min (pressure, *ca.* 350 bar). Column, Nucleosil reversed-phase C_{18}, particle size 5 μm, length 15 cm, I.D. 3 mm. Injection, 30 μl (loop) of 0.6 mg/ml ergotamine. Detection, UV at 254 nm. Peaks: 1 = lysergic acid; 2 = isolysergic acid; 3 = lysergic acid amide; 4 = isolysergic acid amide; 5 = aci-ergotamine; 6 = aci-ergotaminine; 7 = ergotamine; 8 = ergotaminine.

The corresponding retention times and the reproducibilities of retention data (Fig. 6) are shown in Table II. It is therefore not surprising that good reproducibilities are also observed for the quantification of the peaks (Table II). These results compare favourably with those published earlier[6].

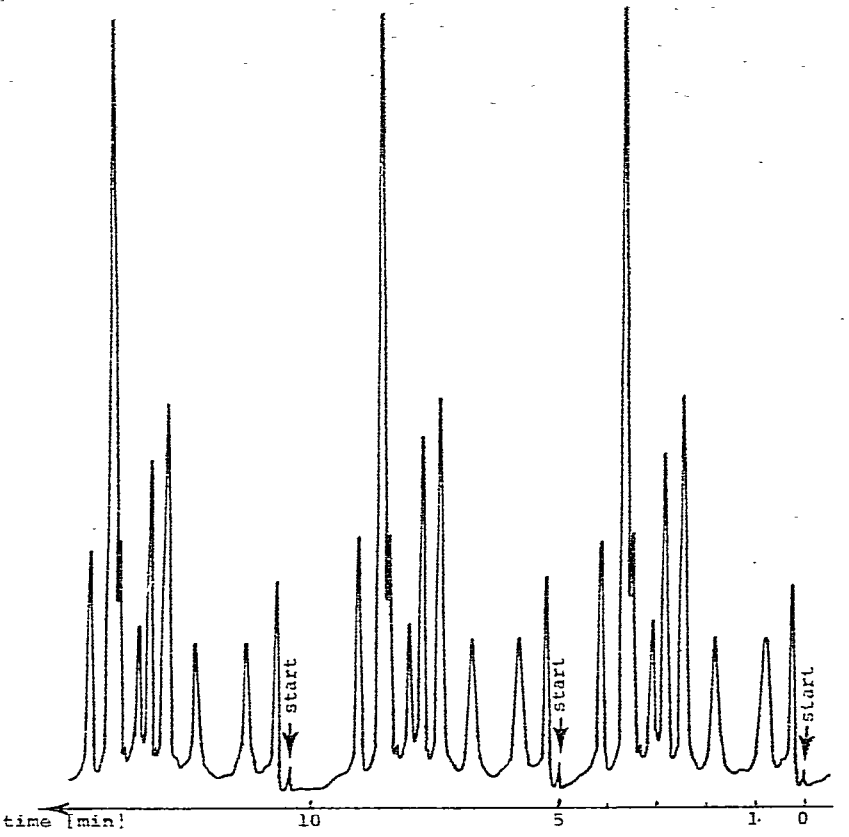

Fig. 6. Separation of ergot alkaloids. Mobile phase programme [acetonitrile–0.01 *M* $(NH_4)_2CO_3$ in water]: step 1, 8%, before injection; step 2, 15%, 32 sec; step 3, 30%, 32 sec; step 4, 40%, 32 sec; step 5, 50%, 32 sec; step 6, 60%, 62 sec. Flow-rate, 2.5 ml/min (pressure, *ca.* 210 bar). Column, Nucleosil reversed-phase C_{18}, particle size 5 μm, length 7.5 cm, I.D. 3 mm. Injection, 30 μl (loop) of 0.6 mg/ml ergotamine. Detection, UV at 254 nm.

A further decrease in separation time would hardly be possible with the equipment used. At peak intervals of 6 sec or less, the time constants of the detector and recorder (response time) start to contribute significantly to band broadening.

Step gradient selector valve as an injection device

In reversed-phase HPLC, it is possible to inject large volumes of samples with very low concentrations. If the sample is dissolved in water or in a solvent with a higher polarity (lower elution force) than the mobile phase, the peak broadening has been shown[7] to be negligible owing to pre-concentration of the sample on top of the column. With a syringe-type injector, it is possible to introduce samples of up to about 50 μl and with loop injection up to 5 ml. For the determination of samples with very low concentrations (<1 ppb), it is desirable to inject up to 1 l of sample solution. With the step gradient selector valve, it is possible to introduce any volume of sample solution into the chromatographic system by replacing one of the solvent reservoirs with the sample. The injection volume is controlled by a timer of the step

gradient control unit. The reproducibility of the injection volume is as good as the flow constancy of the pump and the reproducibility of the timer. In many instances it is recommended to introduce an additional solvent step with water before the injection of the sample and a solvent step with a solvent of medium polarity between the sample injection and the elution of the substance of interest. With this injection mode, it is possible to determine 2 ppb of the ergot alkaloid dihydroergocornine with a reproducibility of better than 5% (injection volume 165 ml). The detection limit was 0.1 ppb. With the selector valve as injection device, it was possible to introduce up to 500 ml of sample solution into the chromatographic system.

CONCLUSIONS

The advantages and disadvantages of the step gradient system are summarized in Table III.

TABLE III

ADVANTAGES AND DISADVANTAGES OF THE STEP GRADIENT SYSTEM COMPARED WITH CONVENTIONAL TWO-PUMP GRADIENT SYSTEMS

Step gradient system	*Two-pump gradient system*
Advantages	*Disadvantages*
Mixing of any number of solvent components possible	Only mixing of two solvent components possible
Exact and reproducible composition of the mobile phase in all parts of the gradient profile	Exact control of the composition of the mobile phase at the start of the gradient (*e.g.*, 1% of solvent A) is critical
Free from interference of small flow inhomogeneities (*e.g.*, caused by air bubbles)	High standard of flow stability is necessary, especially for the first part of the gradient shapes with small concentrations of solvent A
Simple connection to automatic injection systems with reconditioning gradient if necessary	Connection to automatic injection systems in many systems not possible without additional interface
Low cost	High cost
Disadvantages	*Advantages*
Only step-shape gradients	Linear, convex or concave gradient shapes possible
Flow limitations with slow switching valves	No flow limitations up to maximal pump specification

The step gradient system discussed in this paper can be an attractive and cheap alternative to commercially available gradient systems with two pumps and mixing on the high-pressure side. The total cost of such a commercial gradient system (*i.e.*, Waters, Spectra Physics, Altex) is about $14,000 in Europe; the cost of our gradient system, including motor valve, timers and pump, is about $8,000. Recent tests with cheaper pumps with small dead volumes show that this cost could be reduced by about half. The step gradient is particularly suitable for routine quantitative analysis. Although the statistical data in this paper were obtained by manual injection, we have been able to show that automatic loop injectors can be used conveniently in conjunc-

tion with this system. The applications in this study demonstrate, that contrary to many beliefs, gradient elution can be used as a truly quantitative technique.

With the step gradient selector valve, it is possible to introduce very large volumes of sample without decreasing the resolution. Sample volumes up to *ca.* 1 l can be introduced and concentrations below 1 ppb can be detected. Further studies are in progress.

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