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Pressurized gradient electro-high-performance liquid chromatography

Beate Behnke, Ernst Bayer*

Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany

Abstract

A microbore liquid chromatographic system was developed for gradient elution using capillary columns with inner diameters of 50 and 100 μm. In addition, voltage gradients up to 30 000 V can be applied across the length of the column. The dramatic improvement of reversed-phase separations of charged analytes is demonstrated by a separation of detritylated oligonucleotides on 5 μm C_{18} reversed-phase silica gel. Several examples are given, illustrating the influence of applied voltage gradients up to 400 V/cm upon the separation in both isocratic and gradient elution modes.

1. Introduction

Liquid chromatography in capillary columns is a fast developing technique offering the potential of increased performance in comparison to the well established wide-bore methods. Three modes can be distinguished: conventional pressure-driven chromatography [1–4], electroosmotic flow-driven electrochromatography [5–8] and combinations of these [9–11]. The use of packed and open-tubular columns in all three modes has been investigated. Electroosmotic flow arising from an electrical field provides eluent transport with a nearly flat flow profile thus increasing the efficiency. In addition, the electroosmotic flow is independent of the particle diameter, as has been experimentally verified for particle diameters as small as 1.5 μm in packed capillary columns of up to 0.6 m length. The resulting very high efficiency in chromatographic performance has recently been demonstrated [7,8]. The combination of pressure and voltage gradients has been investigated by Tsuda [11], who showed that bubble formation could be diminished by applying pressure to the system.

So far all electrochromatographic techniques use the isocratic approach with capillary columns with very small inner diameters. However, gradient elution is a necessity for many HPLC separations and restriction to isocratic separations greatly limits the range of practical applicability. We have developed a gradient system for liquid chromatographic techniques with packed columns of 50 to 100 μm inner diameter and evaluated the performance of this system in electro-HPLC.

2. Experimental

2.1. Preparation of slurry-packed capillary columns

Fused-silica capillaries of 50 and 100 μm I.D. and 360 μm O.D. were obtained from Polymicro

* Corresponding author.

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Technology (Phoenix, AZ, USA). The frits were prepared by sintering 5-μm spherical silica gel (Gromsil; Grom, Herrenberg, Germany) using a self-made heater. For the packing procedure of either silica gel or reversed-phase material, a slurry of 5-μm particles (Gromsil ODS-2, Grom) in acetone (1:10, w/v) was ultrasonicated for 5 min and transferred to a stainless-steel slurry chamber (25 mm × 1 mm I.D.). The capillary protruded about 5 mm into the chamber which was placed into an ultrasonic bath during packing. The slurry was pumped into the capillary at 400 bar using a liquid chromatographic pump (Model S1100; Sykam, Gilching, Germany).

The production of a packed capillary column proceeded as follows. First a temporary end-frit was prepared by tapping the capillary into silica gel wetted with water, drying and heating. The capillary was then packed with silica gel. The outlet frit was formed by sintering at a distance of approximately 20 cm from the end of the capillary, after which the end-frit was cut off and the capillary emptied on both sides of the frit by flushing with water under ultrasonication. After packing the column with the 5-μm reversed-phase particles ca. 1 cm stationary phase was removed by heating the tip of the capillary, thus causing rapid solvent evaporation and ejection of slurry. Finally this end of the capillary was filled up with silica gel and an inlet-frit sintered after drying.

2.2. Apparatus

In order to use the electrochromatographic system in the gradient mode a modular capillary electrophoresis system (Grom) was combined with a gradient HPLC system (Sykam) as shown in Fig. 1. A stainless-steel six-port rotary valve including an injection port (Rheodyne, Cotati, CA, USA) was used for sample injection. The splitting of eluent was achieved by a stainless-steel T-piece connected to a narrow-bore microparticle-packed column functioning as a flow resistance. The splitter was grounded to protect the pump from any damage that may be caused by the high voltage. The electrolyte block on the inlet side of the capillary column was connected

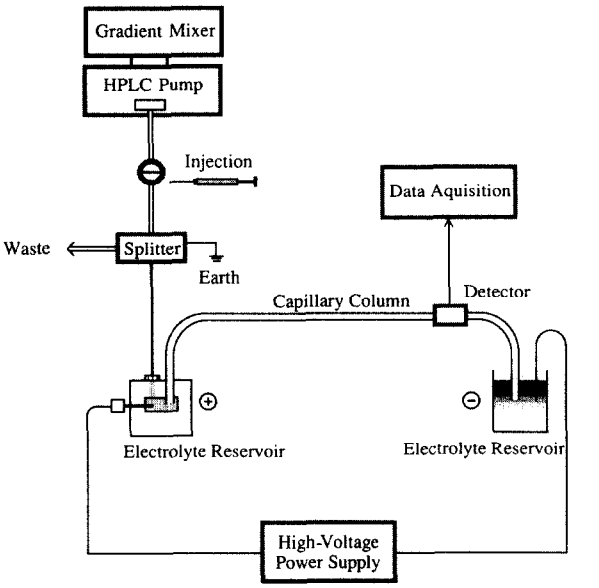

Fig. 1. Schematic representation of an electrochromatographic system.

to the splitter by a laboratory-made interface. A Chromatopac C-R6A (Shimadzu, Kyoto, Japan) was used for data processing.

2.3. Electrochromatography

The chromatographic columns of 50 cm overall length were packed with 5-μm reversed-phase particles to a length of 30 cm. Removal of the polyimide coating allowed on-line UV absorbance detection (260 nm) at 30.2 cm. The flow-rate was 0.5 ml/min at 200 bar. The mobile phase used under isocratic conditions was acetonitrile–buffer containing 10 m*M* triethylammonium acetate (TEAA) (12:88). In the gradient elution mode the buffer was 10 m*M* ammonium acetate at pH 8 containing (A) 0% and (B) 10% acetonitrile. The gradient was 0 to 100% B in 30 min. The buffers were prepared from double-distilled water and thoroughly degassed. The pH was adjusted with a 0.01 *M* triethylamine solution.

Two modes of injection were used. By injection of the sample into the rotary valve only a small fraction entered the chromatographic column after splitting. A second approach was accomplished by filling the electrolyte reservoir

on the inlet side of the capillary with 5 μl sample and pressurizing for 15 s at 200 bar. The reservoir was then flushed with eluent. The concentrations of deoxyoligonucleotides were 5 mg/ml in the split injection mode and 300 μg/ml by injection directly from the electrolyte reservoir.

2.4. Micellar electrokinetic capillary chromatography (MECC)

For electrophoresis the modular capillary electrophoresis system (Grom) was used. Capillaries of 50 μm I.D. and 360 μm O.D. (Polymicro Technology) were flushed for 10 min with 1 *M* NaOH, water and buffer. Overall length of the capillaries was 0.7 m with on-line UV absorbance detection at 0.5 m. The buffer for MECC consisted of 5 m*M* tris(hydroxymethyl)aminomethane, 5 m*M* borate, 7 *M* urea and 50 m*M* sodium dodecyl sulfate at pH 8.9 (HCl). Sample solutions were injected by hydrostatic loading (30 s, 0.1 m). Electrophoresis was performed at 17 kV and 20 μA.

2.5. Chemicals

Oligodeoxyribonucleotides were synthesized on an ABI 38m0B DNA synthesizer using the phosphoramidite approach. The chemicals were purchased from MWG-Biotech (Ebersberg, Germany), Roth (Ulm, Germany) and Merck (Darmstadt, Germany). All chemicals for buffer preparation were of research grade (Merck).

3. Results and discussion

3.1. Microbore HPLC system

Packed fused-silica capillaries used as chromatographic columns provide several advantages. The high mechanical strength allows inlet pressures of up to 800 bar so that long columns can be packed with particles of smaller diameters down to 1.5 μm and used in chromatography [7,8]. Due to the flexibility of the fused-silica capillaries even very long columns can be handled easily. The good optical transparency allows on-column detection in the UV–Vis absorption range. The most important feature for their optional use in electro-HPLC is the isolating property of the fused silica.

A microbore HPLC system was constructed combining a capillary electrophoresis system with an HPLC pump and gradient mixer. The use of capillary columns with inner diameters of 50 to 100 μm demanded a solvent splitter.

Two different injection modes were developed. Split injection via a rotary valve was reproducible but was wasteful of analyte because only a small fraction of the sample volume entered the capillary after splitting. A better approach was the direct injection of sample into the electrolyte reservoir followed by pressurization of the chamber for several seconds. This proved to be an appropriate injection mode although some difficulties were encountered in reproducing the injected amount of the sample.

The development of an interface with minimized dead volume between the HPLC system and the electrolyte reservoir allowed micro-HPLC separations in the gradient mode with columns of 50 μm I.D. While isocratic separations are limited in their application range, the gradient mode offers the tremendous advantage of tuning the selectivity by eluent composition in the same separation. The gradient elution mode also offers the opportunity of easy access to sample enrichment.

This microbore HPLC system allows the scale-down of well established HPLC separations to micro dimensions. In addition, the advantages of plug flow, characteristic of electroosmotically driven systems, upon the chromatographic efficiency can be realized.

3.2. Micro-HPLC separation of oligonucleotides

A comparison of the different modes of micro-HPLC is made on the basis of the separation of oligonucleotides. With isocratic micro-HPLC using 12% acetonitrile only the 9- and 10-mers of a mixture of 1–10-mers could be separated (Fig. 2A). Using 14% acetonitrile the 10-mer oligonucleotide mixture was not retarded at all, while at 10% acetonitrile it was not eluted within 2 h.

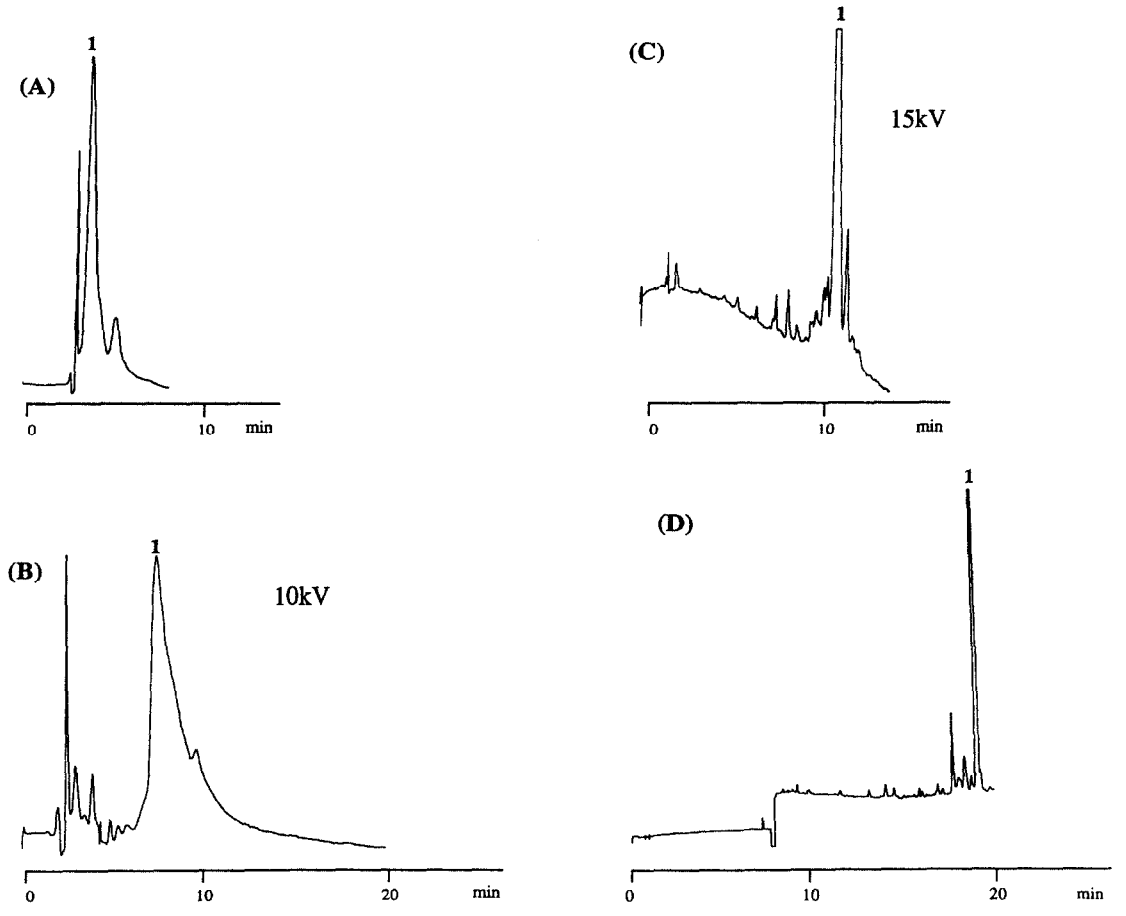

Fig. 2. Comparison of the different modes of micro-HPLC and MECC in analysis of a 10-mer oligonucleotide d(GATGCATAGG–OH) and its by-products. (A) Isocratic micro-HPLC; (B) isocratic electro-HPLC, applied voltage 10 kV; (C) gradient electro-HPLC, applied voltage 15 kV; (D) MECC. Conditions as described in the Experimental section. Peak 1 is the main synthesis product d(GATGCATAGG–OH), all other peaks are by-products.

A variation of the concentration of acetonitrile in the eluent mixture by only 4% under isocratic conditions had a great effect on the selectivity. However it did not result in a decent separation. By ways of contrast, the separation of the mixture of 1–11-mers was easily obtained in the gradient mode (Fig. 3A) thus demonstrating the advantages of the gradient system.

3.3. Electro-HPLC

Electro-HPLC is the combination of microbore HPLC with an applied voltage gradient. Depending on the electric field strength, electroosmotic flow can occur and charged analytes show electrophoretic behavior. There are several advantages arising from the combination of pressure and high voltage. The applied pressure provides stability and reproducibility of the chromatographic performance and prevents bubble formation. Therefore one of the major drawbacks of electrochromatography solely driven by electroosmotic flow, is diminished. Even if some bubbles are formed they are simply swept out thus preventing breakdown of the electric current. Columns with inner diameters up to 150

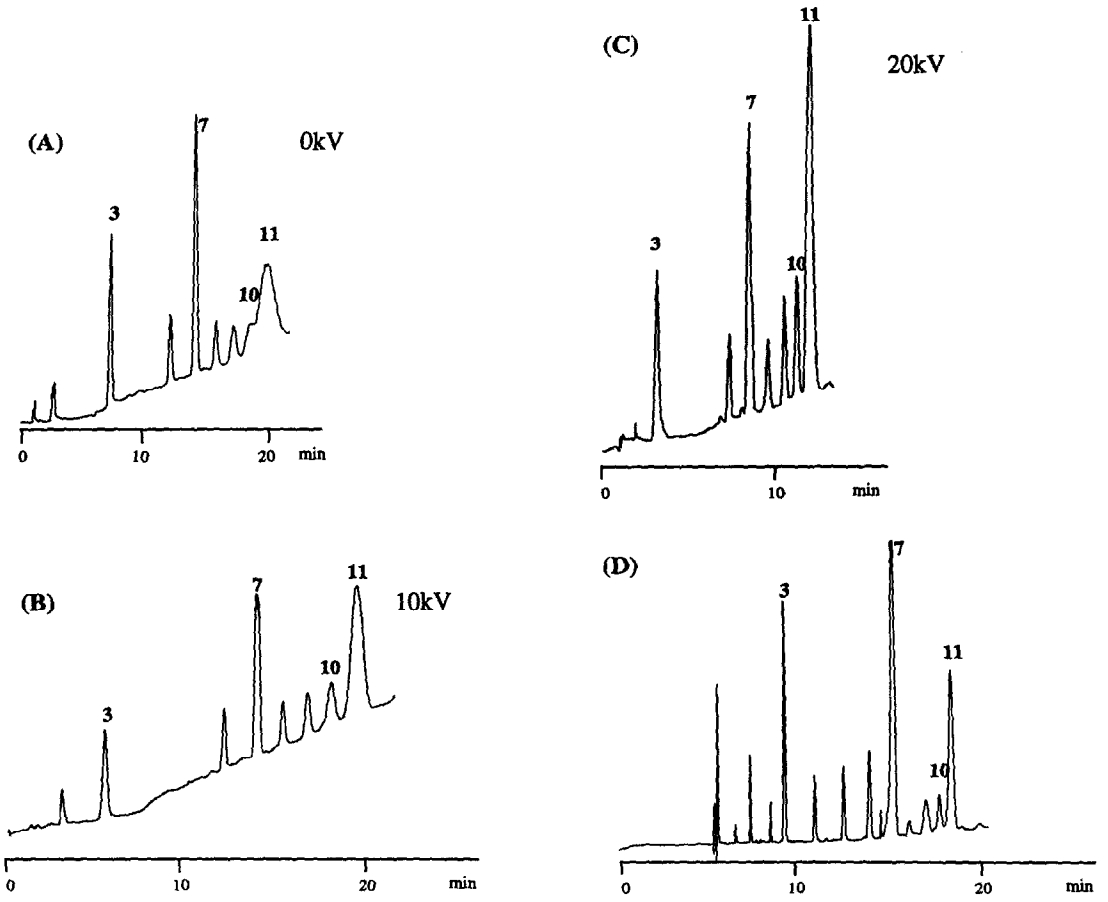

Fig. 3. Influence of applied voltage on the chromatographic separation of oligonucleotides (dC_3–dC_{11}) and comparison with MECC. (A) Gradient micro-HPLC; (B) electro-HPLC, applied voltage 10 kV; (C) electro-HPLC, applied voltage 20 kV; (D) MECC. Conditions as described in the Experimental section. Peaks 3, 7, 10 and 11 correspond to dC_3, dC_7, dC_{10} and dC_{11}, respectively.

μm can be used with a field strength of 400 V/cm in the pressurized system providing high loading capacity and detection sensitivity.

Applying a high-voltage gradient to the chromatographic column results in electroosmotic flow. Its contribution to the overall velocity of the eluent increases with the electric field strength. Using this technique analysis time is shortened and efficiency increases dramatically.

Electric field strength, direction of the voltage gradient, pH, applied pressure, eluent composition and gradient are the most important among various parameters that can be varied to optimize the performance of this highly selective and efficient separation technique.

3.4. Electro-HPLC of oligonucleotides

The higher oligonucleotide homologues in the range 1–11-mer have increasing capacity factors in reversed-phase chromatography and increasing electrophoretic mobility. The direction of the electric field can be used to retard the elution of the higher homologues in order to optimize the selectivity of the separation.

With an electric field strength of 200 V/cm

(Figs. 2B and 3B) the contribution of the electroosmotic flow to the overall velocity was still negligible compared to the pressurized flow (Figs. 2A and 3A). The isocratic electro-HPLC analysis of a 10-mer oligonucleotide mixture (Fig. 2B) revealed the improved separation due to the upstream migration of the analyte molecules resulting in longer retardation and higher selectivity in comparison to the mere HPLC separation (Fig. 2A).

The gradient electro-HPLC separations of an oligonucleotide mixture (dC_3–dC_{11}) shown in Fig. 3B and C demonstrated the optimization of efficiency and speed with increasing participation of electroosmotic flow. Applying a voltage gradient improved drastically the efficiency of the gradient microbore separation of a 10-mer oligonucleotide (Fig. 2C).

These separations were compared with MECC. As in electro-HPLC, MECC discriminates between analytes by a combination of both chromatographic and electrophoretic mechanisms [12]. Both methods show similarly excellent resolution and selectivity (Figs. 2D and 3D), above that which can be accomplished by micro-HPLC.

4. Conclusions

Electro-HPLC combines the advantages of the two basic analytical techniques, chromatography and electrophoresis. The excellent selectivity of gradient-mode chromatography adds to the high efficiency of electrophoretic separations. Using supplementary pressure stable flow conditions are readily obtained, overcoming one of the major problems of chromatography solely driven by electroosmotic flow. Electro-HPLC provides an easy access to dramatically improved HPLC separations optimized in resolution and analysis time by simple scale-down to a micro system and application of a high-voltage gradient.

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