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Pseudo-electrochromatography–mass spectrometry: a new alternative[a]

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ABSTRACT

Pseudo-electrochromatography (pEC) is a separation method that is based on the combination of the chromatographic and electrophoretic behaviour of compounds. This combination enables tuning of the selectivity without changing the composition of the mobile phase. The loadability of pEC is considerably higher than for capillary electrophoresis, which makes the coupling to mass spectrometry very attractive. The applicability of the method was examined for some nucleotides, alkaloids and an antiviral drug as model compounds. The method appeared to be able to replace a modifier gradient elution in reversed-phase systems, thus circumventing the use of an expensive gradient system. pEC has been combined with continuous-flow fast atom bombardment mass spectrometry, as is demonstrated with some examples showing the improvement in the performance of the total system.

INTRODUCTION

Continuous-flow fast atom bombardment (CF-FAB) has become a valuable technique for interfacing different separation techniques with mass spectrometry (MS) [1]. This is due to the good performance of this type of interface, permitting FAB ionization in mass spectrometry combined with liquid chromatography (LC–MS) and capillary electrophoresis (CE–MS) [2]. With growing interest in the analysis of compounds of high molecular weight such as peptides, (glyco)proteins and (oligo)-nucleotides, separation techniques that permit the analysis of these polar and often charged compounds are needed.

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Typical flow-rates allowable in CF-FAB-MS are in the range 5–15 μl/min, implying that conventionally dimensioned high-performance LC (HPLC) is not directly compatible with this type of interface. Two approaches are often applied *i.e.*, the postcolumn splitting of the effluent stream in order to achieve the required flow-rate reduction and the application of miniaturized separation systems. In principle, both approaches result in similar analyte mass flows, meaning that the detection limits obtained will not differ significanlty. A third approach is the so-called phase system switching (PSS) [3]. In this technique, the compound of interest is trapped on a small column, and is subsequently eluted with an appropriate solvent. PSS allows the use of LC systems that are not compatible with (CF-FAB-)MS [4]. Although PSS offers detection limits superior to those given by the other approaches, *i.e.*, post-column splitting and miniaturized chromatography), the technique has the severe limitation that PSS is a target compound analysis approach, which means that the whole procedure has to be focused on one particular compound.

Another separation technique that shows promising results in combination with MS is CE. CE is a miniaturized separation technique that is based on the differences in the electrophoretic mobilities of charged compounds. The compounds are separated in a capillary filled with the electrophoresis buffer by the application of a high voltage over the length of the capillary. Charged compounds will migrate towards the electrode with opposite charge. The electrophoretic migration rate is determined by the charge and the size of the molecules and is proportional to the electrical field strength. In practice, mainly fused-silica capillaries are used, in which a so-called electroosmotic flow (EOF) is generated. This EOF results in a migration of all compounds in the direction of the cathode irrespective of their charge. Owing to the presence of the EOF, negatively and positively charged compounds can be separated in the same run. On the other hand, the length of the capillary needed for a certain separation has to be increased when high EOFs are used.

The efficiencies obtained in CE are far superior to those in other separation methods. Theoretical plate numbers range from 10^5 to $5 \cdot 10^6$ in comparison with 10^4 in chromatography. The use of narrow-bore fused-silica capillaries results in peak volumes that are of the order of only tens of nanolitres. As a consequence of the high efficiencies and the speed of the separation process, mass fluxes are relatively high, which, in principle, is favourable for a mass flow-sensitive detector as a mass spectrometer. Unfortunately, a high mass flux does not always mean a high mass flow. Especially in CE the resulting mass flow is limited owing to the small dimensions of the separation capillary applied. Typical inside diameters are in the range 25–100 μm, and the lengths are commonly between 25 and 100 cm. The flow-rate caused by electroosmosis is only a few hundred nanolitres per minute, which is too low for direct coupling with the CF-FAB interface. By adding a so-called make-up flow to the CE effluent, the final flow-rate can be adjusted to the desired 5–15 μl/min. In addition, this make-up flow can also be used for providing a suitable FAB matrix.

A technique that combines the features of HPLC and CE should overcome the limitations with respect to the flow-rate in CE and the efficiency in HPLC. Therefore, the potential of the combination of (miniaturized) LC and CE and its combination with CF-FAB-MS have been investigated. In fact, electrically driven chromatography or electrochromatography (EC), as described by Knox and Grant [5], is a similar approach, but as the flow in EC is only based on electroosmosis, the resulting flow-

rate is comparable to those obtained in CE. The selectivity in EC is based on the difference in the distribution of the compounds between the stationary and the mobile phases. The driving force is the EOF, which replaces the hydrodynamic flow caused by pressurizing the column in conventional chromatography. In so-called pseudo-EC (pEC), the flow is the combination of a hydrodynamic flow and the electroosmotic flow. The overall migration of the analytes in pEC is the resultant of the chromatographic migration and the electrophoretic migration, as described by Tsuda [6].

The aim of this study was to explore the potential of pEC as an alternative to miniaturized HPLC and to CE as a separation method, offering an additional tuning of the selectivity and suitable for combination with MS detection. Some examples are given to demonstrate the potential of pEC.

EXPERIMENTAL

Chromatography

An outline of the chromatographic system is shown in Fig. 1. The solvent-delivery system consisted of a Model 2150 HPLC pump in combination with a Model 2152 HPLC controller and a low-pressure gradient mixer (all from LKB, Bromma, Sweden). The capillary column was a laboratory-packed 200 mm × 220 μm I.D. fused-silica column packed with Nucleosil 100-5C_{18} (Macherey, Nagel & Co., Düren, Germany). The column effluent was monitored at 260 nm using a Spectroflow 757 variable-wavelength UV detector (ABI Kratos, Ramsey, NJ, USA) equipped with a laboratory-made capillary flowcell using 50-μm fused silica with a volume of about 4 nl. The signal was registered on a Model BD8 multirange recorder (Kipp & Zonen, Delft, Netherlands).

A pre-injector split was applied, as the generation of reproducible and smooth flow-rates appeared possible only at flow-rates above 250 μl/min. The flow was split in the desired ratio using a Swagelok tee (Crawford Fittin, Solon, OH, USA) fitted with a 100 × 3 mm I.D. stainless-steel column manually packed with 8–9-μm XAD-2 packing material as a dummy column. A 100 μm I.D. fused-silica capillary from the gradient system was passed through the splitting tee and the stainless-steel tubing and ended 5 mm short of the injector. Samples were injected with a Model 7413 injector (Rheodyne, Berkeley, CA, USA) fitted with a 0.5-μl sample loop.

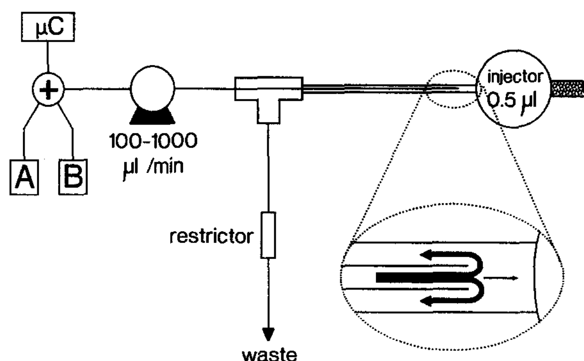

Fig. 1. Scheme of the micro-LC (gradient) system.

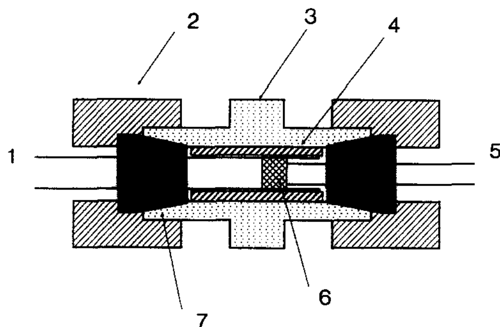

Fig. 2. End-fitting for packed fused-silica column in pseudo-electrochromatography. 1 = 220 μm I.D. packed fused-silica capillary; 2 = 1/16 in female nut; 3 = drilled-through union; 4 = PTFE insert; 5 = 50 μm I.D. fused-silica outlet capillary; 6 = silanized quartz-wool plus; 7 = Vespel ferrule.

For pEC experiments a Model VCS 303-1 reversible-polarity high-voltage power supply (Wallis, Worthing, UK) was used. The injector was connected grounded to earth in order to allow injections during high-voltage operation, while the high voltage was applied to the end of the packed fused-silica column by means of the device depicted in Fig. 2.

Micro-LC–CF-FAB interface

A coaxial [7] and a liquid junction [8] type of interface were employed. For the coaxial interface a 220 μm I.D. fused-silica capillary is inserted into the CF-FAB probe (Finnigan Mat, Bremen, Germany). The 50 μm I.D. detection capillary is run through this sheath capillary 5–50 mm short of the target. This distance was found to be of little importance.

The liquid junction type of interface (Fig. 3) is in principle based on the liquid junction interface used in previous work for the coupling of CE and CF-FAB-MS [9]. The 75-μm fused-silca capillary used in the CF-FAB probe is polished and inserted 10 mm into a 100 mm × 350 μm I.D. fused-silica capillary, and is fixed with epoxy. The 350-μm capillary is connected to a tee with a Vespel ferrule. The 50-μm capillary coming from the micro-LC system (see above) is passed through the tee and inserted

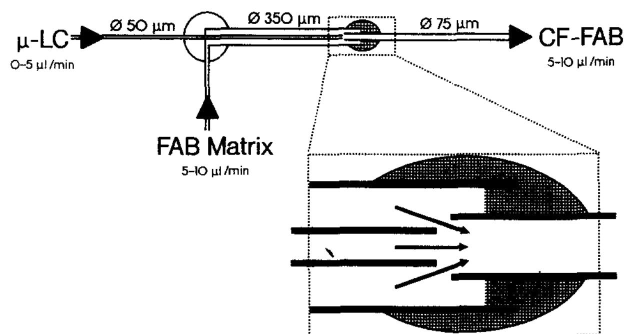

Fig. 3. Schematic diagram of the liquid junction micro-LC–CF-FAB interface for postcolumn FAB matrix addition. Ø = Diameter.

in the 350-μm capillary until positioned to the 75-μm capillary. Delivery of the FAB matrix is performed with a dual syringe pump (Model System 2; ABI Kratos) capable of precise pumping of flow-rates of 1–1000 μl/min. The make-up flow containing 15% of glycerol was applied at flow-rates between 5 and 10 μl/min. The total flow-rate going to the mass spectrometer was in the range 7–14 μl/min.

Mass spectrometry

The CF-FAB probe was mounted on a Finnigan MAT 90 doubly-focusing mass spectrometer equipped with a cryogenic pump at the ion-source housing. A saddle field gun (Ion Tech, Teddington, UK) was used to generate 7-kV xenon atoms. A gold-plated target was used in combination with a wick of pressed paper at the bottom of the ion volume to ascertain stable ionization conditions. Scans from m/z 100 up to 1200 were made at 3 s per decade (cycle time *ca.* 3.5 s) at a resolution of slightly more than 1000. The mass spectrometer was operated in both negative and positive ion mode.

Materials

Analytical-reagent grade methanol (Merck, Darmstadt, Germany) was used. Water was prepared from demineralized water with a GFL Bi-Dest 2108 distillation apparatus (GFL, Hamburg, Germany). Nucleotides were purchased from Boehringer (Mannheim, Germany), 98% chemically pure glycerol from Lamers & Pleuger ('s Hertogenbosch, Netherlands) and trifluoroacetic acid (TFA), dibutylamine (DBA) and ammonia (25%) from Merck.

Preparation of micro-columns

Pieces of 220-μm fused-silica capillary (SGE, Melbourne, Australia) of the desired length were cut and both ends were polished. One end of the capillary was connected to a male union (Swagelok) containing a metal frit (Alltech, Deerfield, IL, USA). Methanol was used as the packing solvent, and was delivered by an air-actuated pump (DSHF-302; Haskell, Burbank, CA, USA). The slurry reservoir was a 2-ml magnetically stirred high-pressure mixing chamber. In order to prevent the fused-silica capillary from exploding activation of the packing pump, a needle valve was installed between the slurry reservoir and the pump.

The packing procedure is as follows. Air is removed by flushing the system with methanol. The needle-valve is closed and a pressure of 50 bar is built up. Slurries are prepared by dispersing 250 mg of the packing material in 25 ml of methanol. This slurry is ultrasonically homogenized for 5 min and 2 ml are introduced into the slurry reservoir by means of a syringe. After connecting the fused-silica capillary to the slurry reservoir, the stirrer is activated, the needle valve is opened and the pressure is rapidly increased to 400 bar. Pressure is applied for 1 h, after which the needle valve is closed. The system is left to depressurize for 30 min and the column is disconnected. After packing, the column is primed with water and the column ends are sealed with septa.

Prior to use of the micro-column, 5 mm of the packing at the column end is removed, a 1-mm plug of silanized quartz-wool is introduced and a 50 μm I.D. fused-silica capillary is inserted in the micro-column. A piece of PTFE tubing (10 mm × 1/16 in. O.D. × 0.35 mm I.D.) is slid over this connection and inserted in a

drilled-through Swagelok male union and fixed with two Vespel ferrules, as shown in Fig. 2. The advantage of this type of connector over glueing is that columns can be changed without reinstalling the 50-μm detection capillary. Severe deterioration of plate numbers has not been observed with this construction.

The top of the column is connected to the injection valve by means of a Rheodyne nut, a Vespel ferrule and a PTFE liner (4 mm × 1/16 in O.D. × 0.35 mm I.D. tubing) to fill up the void space [10]. The described packing procedure is reproducible, and results in plate numbers for polycyclic aromatic hydrocarbons of about 12 000 for a 200-mm column. Further optimization of the procedure should improve this.

RESULTS AND DISCUSSION

Chromatographic phase systems that are applied for the separation of charged compounds are mainly based on the use of a counter ion either for ion-pair formation or as a competing ion in an ion-exchange system. As the coupling with MS requires that additives to the mobile phase are volatile, the number of applicable counter ions is drastically decreased. Commonly, strong acids are used to provide an anionic counter ion and quaternary amines to provide cationic counter ions. Volatile secondary and tertiary amines appear to be a good alternative to the non-volatile quanternary ammonium compounds. This study was limited to compounds that can be chromatograped in ion-pair reversed-phase systems. Several test mixtures have been used for the investigation of pEC.

Fig. 4a shows the chromatogram of a test mixture of some alkaloids run under isocratic conditions. Alkaloids are basic compounds that are neutral at high pH

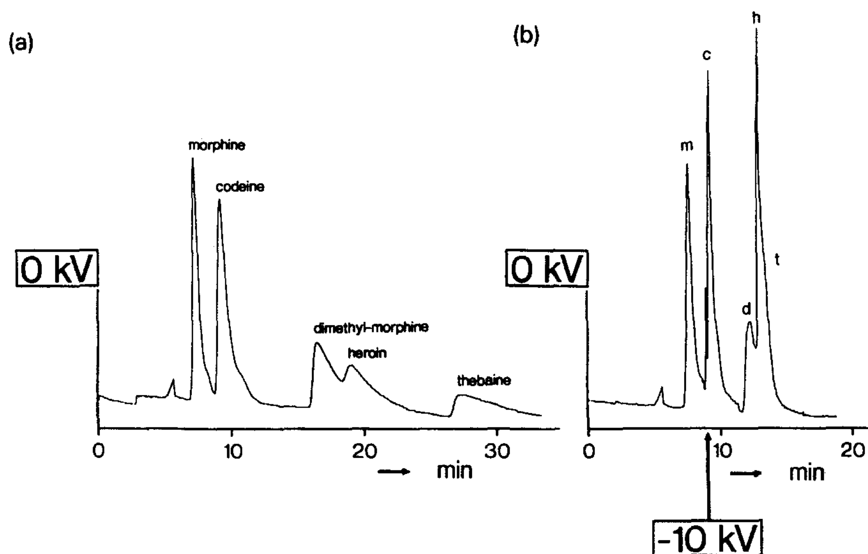

Fig. 4. Chromatograms of a test mixture of some morphine alkaloids. Column, 150 mm × 220 μm I.D. packed with 5-μm Nucleosil 100 C_{18}; mobile phase, acetonitrile–2 mmol/l ammonium acetate (2:3, v/v); applied voltage, 10 kV starting at time t = 9 min; UV detection at 220 nm, 0.01 a.u.f.s.; amounts injected, *ca.* 50 ng of each compound.

values ($pK_a > 9$). Although good efficiencies were obtained for neutral compounds, the charged alkaloids showed severe tailing, resulting in a disappointing performance of the separation system. The use of an end-capped packing material will improve the performance, but this study was not focused on the optimum separation of this type of compound. By applying a voltage over the separation column an electrophoretic component will be superimposed on the chromatographic migration. Fig. 4b shows the chromatogram of the same test mixture, but under pEC conditions. It is clear that the application of a voltage over the capillary influences the separation characteristics considerably.

An even stronger example is given by the separation of a test mixture of some nucleotides. Nucleotides are charged polar compounds possessing different numbers of phosphate moieties, which make them very well suited for separation techniques in which the electrophoretic mobility is involved.

Although much effort has been put into analyses based on MS using flow injection as an introduction technique applying different types of interface [11–16], to our knowledge no systems for nucleotide analysis by LC–MS have been reported. For UV detection-based analysis, Willis *et al.* [17] studied the use of triethylamine as an "ion-pairing" agent for the separation of a large number of nucleotides and related compounds. With respect to volatility, triethylamine looks very promising, but in practice it turned out that the concentration must be above 10 mmol/l in order to achieve sufficient retention of nucleotides. The use of such high TEA concentrations causes excessive ion-source contamination while at same time various chromatographic parameters such as selectivity and resolution are substantially affected. Haastert [18] described the use of various alkylamines for the separation of cyclic nucleotide derivatives, and reported the influence of the alkyl chain length, the number of alkyl chains, the pH of the mobile phase and the concentration of the alkylamine in

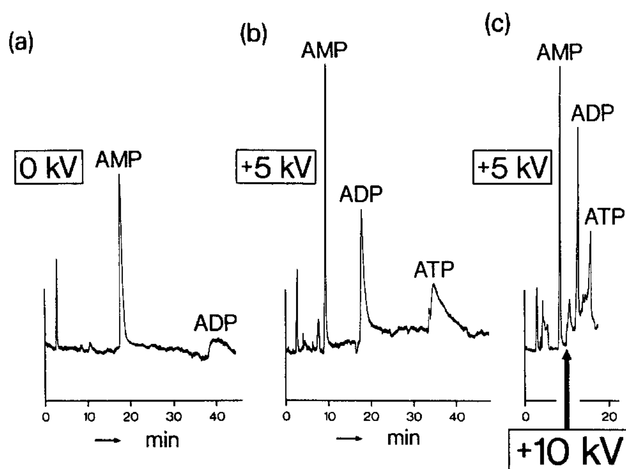

Fig. 5. Separation of 17 ng of AMP, ADP and ATP. Column, 200 mm × 220 μm I.D. packed with 5-μm Nucleosil 100C_{18}; mobile phase, 10% methanol in 2 mmol/l dibutylamine at pH 5.0; UV detection at 260 nm, 0.0025 a.u.f.s. (a) Isocratic conditions; (b) applied voltage +5 kV; (c) starting voltage +5 kV, increased to +10 kV at $t = 9$ min.

question. The longer alkyl chains of dibutylamine (DBA) gave indeed sufficient retention of nucleotides even when used at concentrations as a low as 1–2 mmol/l.

Fig. 5a shows the chromatogram of three adenosine phosphates, separated under isocratic conditions. Retention was obtained, but ADP and ATP are eluted with a poor performance. In Fig. 5b the chromatogram of the same mixture is given, but in this case a voltage of + 5 kV was applied to the downstream electrode, implying that the anionic compounds are accelerated. Although the symmetry of the later eluting compounds is far from ideal, it can be seen that a real peak compression takes place. Nevertheless, it must be realized that the same effect should be obtained by applying solvent-generated gradient elution by increasing the content of modifier in the mobile phase. In that event, however, the compatibility with the CF-FAB ionization may be diminished as the FAB conditions are affected by the change in the mobile phase composition.

The influence of the application of a voltage over the capillary is even clearer in Fig. 5c, in which after the elution of AMP the applied voltage of + 5 kV was increased to + 10 kV. It can easily be predicted that by using voltage-programmed elution, further optimization of the performance can be obtained. This aspect is currently under investigation.

Fig. 6 represents the mass chromatograms of inosine-5*k*′-triphosphate (ITP), (a) under isocratic conditions and (b) under pEC conditions. These data were obtained by applying MS in the negative-ion CF-FAB (NI-CF-FAB) mode. The signal-to-noise ratio is improved by a factor of about 10. In both instances the amount injected was about 50 ng, implying that 10–50 pmol amounts are needed for the full-scan detection of this nucleotide.

Fig. 7 gives another example of the potential of pEC, showing the mass chromatograms of suramin. Suramin is an antiviral drug with six sulphonate groups, which means that the compound will be in the form of a 6− anion that will be strongly influenced by an electric field. Fig. 7a shows the result obtained with a modifier gradient and Fig. 7b was obtained using pEC. The theoretical plate numbers of 5000 and 34 000, respectively, demonstrate an improvement that cannot be due only to an on-column concentration caused by isotachophoresis effect. With injection of an analyte dissolved in a buffer with an ionic strength below that of the running buffer, a discontinuous buffer system is used, which leads to peak sharpening during

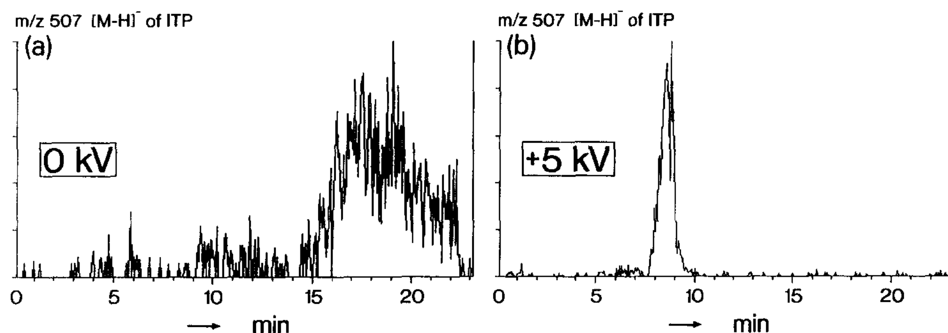

Fig. 6. Mass chromatograms of the $[M-H]^+$ ion of 50 ng of ITP obtained by (a) isocratic chromatography and (b) pEC. Conditions as in Fig. 5.

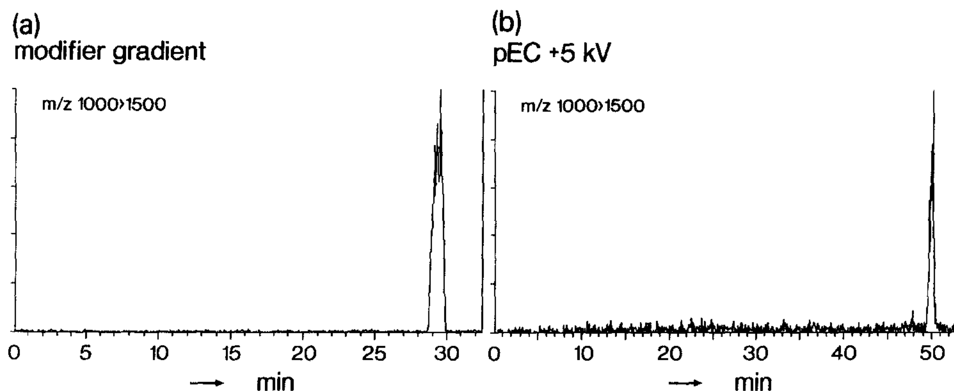

Fig. 7. Summed mass chromatograms (m/z ranging from 1000 to 1500) of 250 ng of suramin (MW 1296). Conditions as in Fig. 5. (a) Modifier gradient (5–60% methanol in 30 min); (b) pEC, modifier content 30%, applied voltage 5 kV.

the injection [19,20]. Such a compression results in a negligible injection volume, meaning that the peak broadening should only be caused by the separation processes and the detection. Taking these aspects in consideration, the obtained plate number of 34 000 is still better than expected and cannot be fully explained. A more systematic study to the peak broadening is in progress.

Fig. 8a shows the pEC–CF-FAB-MS results obtained by adding the $[M-H]^-$ ion of uridine disposphate (UDP, m/z 403) and uridine triphosphate (UTP), m/z 483). The negative-ion FAB mass spectra were obtained after injection of 10 ng of both compounds into the pEC system.

In the negative-ion mode no nucleotide ions were observed when the matrix contained TFA. However, when DBA was used instead of TFA, intense $[M-H]^-$ ions were observed. Fig. 8b and c show the negative-ion mass spectra of UDP and UTP. Ions at m/z 159 ($[P_2O_6H]^-$), 177 ($[P_2O_7H_3]^-$), 239 ($[P_3O_9H_2]^-$) and 257 ($[P_3O_{10}H_4]^-$) give information about the number of phosphate groups. Similar ions at m/z 97 ($[PO_4H_2]^-$) and 79 ($PO_3]^-$) are expected for the monophosphate nucleosides [21], but were not observed because scans were made starting at m/z 100.

Interface performance

Postcolumn addition allows any FAB matrix to be used without impairing the chromatographic separation process. The only requirement is that this should be done without (significant) decreases in chromatographic resolution. Both the coaxial and the liquid junction type of interface meet this requirement. Nevertheless, the liquid junction was chosen because the coaxial interface had some practical disadvantages, the first being the difficulty of inserting a 200 μm O.D. capillary in a 220 μm I.D. capillary over a length of 75 cm. Owing to small irregularities in the capillaries used this is sometimes impossible, or the sheath capillary is damaged. The second is that the success of this procedure depends heavily on only small between-batch differences in the fused-silica capillaries used.

This postcapillary addition results in dilution of the analytes, but with a mass flow-sensitive detector this will not affect the detection limits.

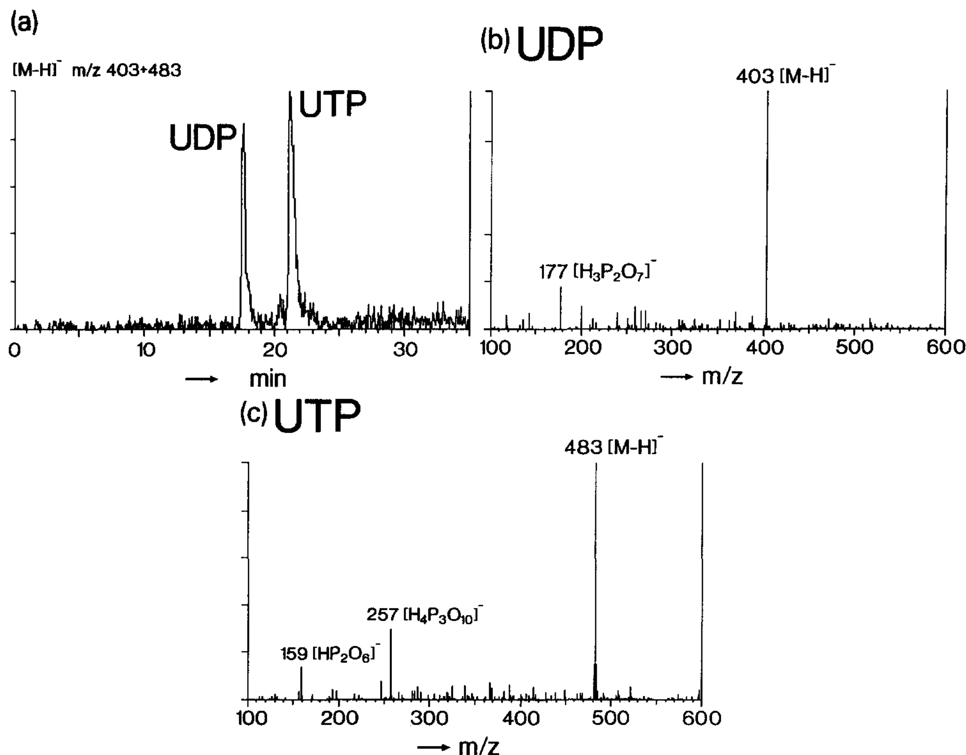

Fig. 8. (a) Mass chromatograms of the $[M-H]^+$ ions of 10 ng of UDP and UTP and (b) and (c) the corresponding negative-ion FAB mass spectra as obtained with pEC. Conditions as in Fig. 5c; applied voltage, starting voltage 5 kV, increased to 10 kV at $t=8$ min.

Limitations

The use of a hydrodynamic flow of several microlitres per minute makes the electroosmotic flow negligible. This means that for neutral compounds pEC cannot be distinguished from capillary chromatograhpy. Further, dilute buffers are required in pEC using 220 μm I.D. columns in order to avoid excessive heat generation within the capillary and simultaneously a greater risk of air bubble formation. Buffer concentrations exceeding 5 mmol/l result in unacceptably high electric currents and lower the performance of the system considerably.

CONCLUSIONS

pEC appeared to be a good supplementary technique for capillary LC. The method is useful for obtaining improved chromatographic performance for ionic compounds. The selectivity in pEC can be tuned easily by voltage programming. pEC appears to be readily compatible with CF-FAB-MS. In the analysis of charged compounds the use of an expensive gradient system can be avoided by the application of pEC. The described system allows the full-scan detection of nucleotides in 10–50 pmol amounts without the necessity for derivatization.

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